Nitrogen Fixation and

Nitrate Assimilation in Soybean

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by

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Declaration

The research described in this thesis is my own work unless acknowledgement is made in the text. It has not been submitted for any other degree.

The following paper was published during the period of study:

Schuller KA, DA Day, AH Gibson, PM Gresshoff 1986 Enzymes of ammonia assimilation and ureide biosynthesis in soybean nodules: Effect of nitrate. Plant Physiol 80: 646-650

Three other papers entitled:

(1) "N_2 and Nitrate Assimilation in a Range of Soybean Genotypes";

(2) "Nitrogen Fixation and Ureide Metabolism in Soybean cv. Bragg and its Mutant Derivative nts382", and

(3) "The Effect of Inoculum Dose and Nitrate Treatment on Nodulation and N_2 Fixation of Soybean cv. Bragg and its Mutant Derivative nts382"

are in preparation.

K. A. Schuller

(Kathryn A. Schuller)

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Abbreviations

ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ARA	Acetylene Reduction Activity
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CHES	2[-Cyclohexylamino]ethane-sulfonic acid
d	day
DAP	Days After Planting
DTE	Dithioerythritol
DTT	Dithiothreitol
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic Acid
FID-GC	Flame Ionization Detection-Gas Chromatography
FW	Fresh Weight
GMP	Guanosine Monophosphate
GOGAT	Glutamine Oxoglutarate Aminotransferase
GS	Glutamine Synthetase
h	hour
β-HBDH	β-Hydroxybutyrate Dehydrogenase
IAA	Indole Acetic Acid
IEF	Isoelectric Focusing
IMP	Inosine Monophosphate
Kd	Kilodaltons
MES	2[N-Morpholino]]ethanesulfonic acid

min	minute(s)
mRNA	messenger Ribonucleic Acid
N	Nitrogen
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NRA	Nitrate Reductase Activity
PAGE	Polyacrylamide Gel Electrophoresis
PEP	Phosphoenolpyruvate
Pi	inorganic Phosphate
PMSF	Phenylmethylsufonyl Fluoride
PRAT	Phosphoribosyl Pyrophosphate Amidotransferase
PVP	Polyvinyl Polypyrrolidone
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
TCA	Tricarboxylic Acid
TEMED	N,N,N,N-Tetramethyl-ethylenediamine
TES	N-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid
XDH	Xanthine Dehydrogenase
XMP	Xanthine Monophosphate

Abstract

This thesis examines the inhibitory effect of nitrate on nodulation and N_2 fixation in soybean. N_2 fixation was assayed either directly using acetylene reduction or $^{15}N_2$ fixation or indirectly from the ureide content of xylem sap. All three methods gave similar results in relation to the inhibitory effect of nitrate on N_2 fixation.

When nitrate was supplied to an established soybean-Bradyrhizobium japonicum symbiosis, there was a rapid (within two days) decline in nitrogenase activity and the ureide content of xylem sap. Nitrogenase activity of bacteroids isolated from the nodules of nitrate treated plants was not inhibited during this period. The in vitro activities of nodule cytoplasmic glutamine synthetase and glutamine oxoglutarate amino transferase (enzymes involved in ammonia assimilation), xanthine dehydrogenase, uricase and allantoinase (enzymes involved in ureide biosynthesis) and invertase, fructokinase and PEP carboxylase (enzymes involved in carbon metabolism) were similarly not affected. Likewise, there was no effect on nodule fresh weight, total soluble protein or leghaemoglobin content of nodules. Analysis of nodule cytoplasmic proteins by polyacrylamide gel electrophoresis failed to reveal any nitrate associated changes in the levels of any of the nodule proteins detected. It was concluded that the primary cause of the nitrate-induced decline in nitrogenase activity did not involve any of the above factors.

A comparison between soybean cv. Bragg, three other soybean cultivars and a supernodulating soybean mutant nts382 (derived from soybean cv. Bragg) indicated that the mechanisms involved in the inhibitory effect of nitrate on nodulation and N_2 fixation may differ from one another. Evidence is presented that competition between nitrate reduction in the leaves and N_2 fixation in the nodules, for available photosynthate, may not be the primary cause of the nitrate-induced decline in nitrogenase activity.

When compared with its parent cultivar Bragg, the soybean mutant nts382 exhibited reduced susceptibility to nitrate-induced inhibition of nodulation and N_2 fixation. Nodulation of nts382 was almost totally unaffected by nitrate, whereas nitrogenase activity of the mutant was somewhat less susceptible to the inhibitory effect of nitrate.

Nitrogenase activity of nts382, expressed on a nodule fresh weight basis, was several-fold less than that of Bragg. This could not be attributed to an impairment in sucrose metabolism in the nodules of the mutant. However, it is likely to be due to the reduced bacteroid protein content of the nodules of nts382. The leghaemoglobin content of nts382 nodules was also reduced.

Whole plant nitrogenase activity of nts382 was not greater than that of Bragg but, despite this, the ureide content of the xylem sap, nodules and leaves of the mutant was several-fold higher. There was no

evidence of elevated ureide biosynthesis in the roots of non-nodulated, nitrate-grown nts382 plants. Neither was there any apparent impairment in ureide utilization in the leaves of symbiotically-grown nts382 plants.

At the high Bradyrhizobium japonicum inoculum dose usually used, nts382 was supernodulating. However, when the inoculum dose was decreased, nodulation of the mutant was reduced to the level of the parent cultivar Bragg. Under these conditions, nodulation of nts382 was still nitrate-tolerant but nitrogenase activity no longer showed reduced susceptibility to nitrate inhibition. These results are discussed in relation to the morphology of nts382 nodules. Under low inoculum conditions, individual nodule mass, nitrogenase activity (per g nodule fresh weight) and bacteroid protein content of the nodules were similar for Bragg and nts382. The ureide content of nts382 nodules declined in response to a reduction in the inoculum dose.

The nodule cytoplasmic protein profiles of Bragg and nts382, as revealed by polyacrylamide gel electrophoresis, were basically similar except for a 24Kd protein which was more abundant in nts382 nodules than it was in Bragg nodules.

The results are discussed in relation to the current hypotheses on the mechanism of nitrate inhibition of nodulation and N_2 fixation.

CHAPTER 1

Introduction

1.1 N_2 and Nitrate as Alternative Nitrogen Sources for Legumes.

Legumes in symbiotic association with Rhizobium (or Bradyrhizobium) bacteria have access to atmospheric N_2 as a nitrogen source. Infection of the roots of legumes by Rhizobium bacteria induces differentiation of the root tissue to form nodules and of the bacteria to form N_2 fixing bacteroids (see Bauer 1981). N_2 fixation, the reduction of N_2 to ammonium, is catalyzed by the bacteroid enzyme nitrogenase.

Nitrogenase

$$N_2 + 12ATP + 6NADPH + 8H^+ \rightarrow 2NH_4^+ + 12ADP + 12Pi + 6NADP^+ + 3H_2$$

The ammonium produced is excreted by the bacteroids into the nodule cytoplasm where it is further assimilated before being exported to other parts of the plant (see Boland et al. 1980). In return the plant supplies the bacteroids with respiratory substrates which are consumed in the production of the large quantities of reducing equivalents and ATP required for nitrogenase activity. The respiratory substrates supplied to the bacteroids are probably carboxylic acids. The evidence for this is two-fold. Firstly, carboxylic acids stimulate nitrogenase activity of isolated bacteroids (Bergersen and Turner 1967, Trinchant et al. 1981, McNeil et al. 1984). Secondly, bacteroids which are either

impaired in carboxylic acid uptake or have a defective TCA cycle form ineffective nodules (see Ronson and Astwood 1985).

The plant also provides an environment, namely the nodule, in which the O_2 concentration in the vicinity of the bacteroids is high enough to support bacteroid respiration but not so high as to damage the O_2 sensitive nitrogenase. The low O_2 concentration in the infected zone of the nodule is maintained by a more of less variable physical barrier to O_2 diffusion and a combination of both coupled and uncoupled bacteroid respiration (see Appleby 1984, Witty et al. 1984). The extent of variability of the barrier and the significance of changes in bacteroid respiration differ between species (Witty et al. 1984). In this low O_2 environment leghaemoglobin, an O_2 binding protein, facilitates the transport of O_2 to the bacteroids (see Appleby 1984).

In common with other plants, legumes can also utilize nitrogen from the soil in the form of ammonium and/or nitrate. When soil nitrogen levels are high, however, infection, nodule formation and N_2 fixation are inhibited and senescence of already established nodules is induced (see Pate and Atkins 1983). The mechanism underlying this effect is not known. Furthermore, different mechanisms may be responsible for the effects on nodule formation and N_2 fixation. Most research has focused on the effect of nitrate rather than ammonium. This is because of its greater abundance in cultivated soils, due to active nitrification.

The first step in the assimilation of nitrate in higher plants is its reduction to nitrite, which is catalysed by nitrate reductase. This

is followed by the nitrite reductase catalysed reduction of nitrite to ammonium (see Pate and Atkins 1983).

Nitrate Reductase

$$NO^-_3 + NAD(P)H + H^+ \text{----> } NO^-_2 + NAD(P)^+ + H_2O$$

Nitrite Reductase

$$NO^-_2 + 6Fd_{red} + 8H^+ \text{---> } NH_4^+ + 6Fd_{ox} + 2H_2O$$

Ammonium might therefore be implicated in the effect of nitrate on N_2 fixation. This is not borne out by experimental evidence, however, since there is no effect of ammonium on the activity of nitrogenase partially purified from Rhizobium lupini bacteroids (Kennedy 1970). Neither is there any effect of ammonium on electron transport of isolated Rhizobium leguminosarum bacteroids (Laane et al. 1980). The latter is in contrast to observations with Azotobacter vinelandii in which ammonium dissipates the electrical component of the proton motive force hence inhibiting nitrogenase activity. It should be noted, however, that bacteroids lack an ammonium uptake system (Bergersen and Turner 1967, O'Hara et al. 1985, Howitt et al. 1986).

Ammonium represses nitrogenase synthesis in free-living N_2 fixing bacteria such as Klebsiella pneumonia (Eady et al. 1978). In contrast, although total protein synthesis is inhibited, there is no specific effect of either ammonium or nitrate on nitrogenase synthesis in bacteroids (Bisseling et al. 1978, Noel et al. 1982). Furthermore, nitrogenase activity of bacteroids isolated from the nodules of nitrate

and/or ammonium treated plants is unaffected despite a marked inhibition of nitrogenase activity of nodulated roots (Houwaard 1979, 1980, McNeil et al. 1984). Obviously, the inhibitory effect of nitrate on N_2 fixation cannot be attributed to an effect of ammonium on nitrogenase per se, electron transport to nitrogenase or the synthesis of nitrogenase proteins.

Another possible mediator of the nitrate effect is nitrite, an intermediate in the reduction of nitrate to ammonium. Nitrite inhibits N_2 fixation by bacteroids isolated from soybean nodules and by nitrogenase purified from Rhizobium japonicum bacteroids (Rigaud and Puppo 1977, Trinchant and Rigaud 1980). It also oxidises leghaemoglobin and consequently inactivates the O_2 binding function of this haemoprotein (Rigaud and Puppo 1977). When compared with nodules containing nitrate reductase expressing Rhizobium strains, nitrite accumulation in nodules containing nitrate reductase deficient strains is much reduced. However, despite this, the degree of inhibition of N_2 fixation is similar (Gibson and Pagan 1977, Stephens and Neyra 1983, Streeter 1985 a, b). It therefore appears that nitrite produced by the bacteroids plays no role in the inhibitory effect of nitrate on N_2 fixation. However, nitrite formed in the nodule cytoplasm via the plant derived nitrate reductase may still be involved.

An alternative explanation for the inhibitory effect of nitrate on N_2 fixation, known as the "photosynthate deprivation hypothesis", postulates that competition between nitrate reduction and N_2 fixation for carbon skeletons and reducing equivalents results in reduced

allocation of photosynthate to the nodules and consequent inhibition of N_2 fixation (Oghoghorie and Pate 1971). This hypothesis is supported by the observed decline in the translocation of ^{13}C- and ^{14}C-labelled photosynthate to nodules following nitrate treatment (Small and Leonard 1969, Latimore and Giddens 1977, Kouchi and Yoneyama 1984 a, b). It is not known, however, whether this is causal or simply the result of the decreased sink capacity of the nodules due to inhibition of N_2 fixation. Recently reported experiments, employing a shorter nitrate treatment period, indicate that the decline in nitrogenase activity precedes the decline in the translocation of photosynthate to the nodules in soybean but not in pea (Wasfi and Prioul 1986).

1.2 Assimilation of Fixed Nitrogen in Legumes Nodules

In legume nodules, ammonium exported by the N_2 fixing bacteroids is initially incorporated into glutamine via the reaction catalysed by glutamine synthetase (GS). This is coupled with the transamination of glutamine to yield glutamate, which is catalysed by glutamine oxoglutarate aminotransferase (GOGAT). Glutamate then undergoes further transamination reactions or is recycled as a substrate for the glutamine synthetase reaction (see Boland et al. 1980).

$$\text{Glutamate} + \text{ATP} + NH_4^+ \xrightarrow{\text{GS}} \text{Glutamine} + \text{ADP} + \text{Pi}$$

$$\text{Glutamine} + \text{2-Oxoglutarate} + \text{NADPH} + H^+ \xrightarrow{\text{GOGAT}} 2\ \text{Glutamate} + NADP^+$$

In the nodules of temperate legumes, such as lupin and pea, glutamate undergoes a transamination reaction, catalysed by aspartate aminotransferase to yield aspartate. Asparagine synthetase subsequently catalyses the formation of asparagine from aspartate and glutamine. Asparagine and to a lesser extent aspartate are the major nitrogen compounds exported from the nodules to the shoots in temperate legumes (see Pate and Atkins 1983).

In tropical legumes, such as soybean and cowpea, on the other hand, the ureides allantoin and allantoic acid are the major nitrogen compounds exported from the nodules (see Schubert and Boland 1984). Ureide biosynthesis in the nodules of these plants involves the oxidative degradation of purines synthesized de novo. The pathway of ureide biosynthesis and the location of the enzymes involved, as proposed by Schubert (1986), is shown in Fig. 1.1. Initially glutamine, aspartate and glycine are incorporated into the purine ring. This occurs in the plastids of the bacteroid containing cells of the nodule. Subsequently, purines are oxidatively degraded to yield ureides. The initial reaction in purine oxidation, the oxidation of xanthine to uric acid, occurs in the cytosol and is catalysed by xanthine dehydrogenase (XDH). However, there is still some debate as to whether XDH is located in the infected cells (Triplett 1985) or in the uninfected cells (Nguyen et al. 1986) of the nodule. As a result of this the identity of the intermediate which passes between the infected and the uninfected cells is not known. The subsequent reactions in purine oxidation are the uricase catalysed oxidation of uric acid to yield allantoin and the allantoinase catalysed conversion of allantoin to allantoic acid. Both

reactions occur in the uninfected cells with uricase being located in the peroxisomes and allantoinase being associated with the endoplasmic reticulum.

In soybean, the relative abundance of ureides (ureides as a proportion of total nitrogen) in the xylem sap and shoot axes has been used as a measure of the relative contributions of N_2 fixation and nitrate assimilation to total plant nitrogen (McClure and Israel 1979, Herridge 1982, 1984). When nitrate is supplied to symbiotically grown soybean plants, the relative abundance of ureides in the xylem sap and shoot axes declines. This is due both to a decline in ureide production and to an increase in total nitrogen resulting from the presence of nitrate and products of its reduction.

As well as being synthesized in the nodules, ureides are also synthesized in the cotyledons and roots of soybean (Polayes and Schubert 1984). However, the ureide content of the xylem sap and above ground parts, is much lower in non-nodulated soybean plants than it is in nodulated plants (McClure and Israel 1979, Streeter 1979). As in the nodules, ureide biosynthesis in young cotyledons (less than 4 d-old and in older roots (greater than 8 d-old) occurs via de novo purine biosynthesis (Polayes and Schubert 1984). In older cotyledons, ureides arise via nucleotide breakdown.

1.3 The Effect of Nitrate Treatment on the Assimilation of Fixed Nitrogen.

The effect of nitrate treatment on some of the enzymes involved in ammonia assimilation and ureide biosynthesis in legume nodules has been investigated in both alfalfa and soybean (Groat and Vance 1981, Becana et al. 1984, Zengbe et al. 1984). Provision of nitrate to an established alfalfa-Rhizobium symbiosis results in a decline in both GS and GOGAT activity (Groat and Vance 1981, Becana et al. 1984). The decline is delayed, however, relative to the decline in N_2 fixation. The duration of the delay and the severity of the decline depends on the concentration of nitrate supplied. Glutamate dehydrogenase, which catalyses an alternative reaction resulting in the incorporation of ammonia into glutamate, becomes more active in nodules following nitrate treatment. This is in contrast to the apparent absence of any role for this enzyme in ammonia assimilation in the nodules of plants dependent solely on N_2 as a nitrogen source.

When nitrate or ammonium is supplied to symbiotically-grown soybean plants from the time of planting, development of XDH, uricase and allantoinase activity is not affected (Zengbe et al. 1984). Interpretation of the results of this study is complicated, however, by the inhibitory effect of combined nitrogen on nodule development.

1.4 Regulation of the Enzymes of Ureide Biosynthesis in Nodules.

Information on the regulation of the enzymes of ureide biosynthesis in legume nodules has recently begun to accumulate. XDH purified from soybean nodules is a molybdoiron flavoprotein with a pH optimum of 7.5 and is inhibited by uric acid and NADH, the products of the reaction it catalyses (Triplett et al. 1982, Boland et al. 1983). Uricase purified from soybean nodules has a pH optimum of 9.5 and is a dimer at this pH (Lucas et al. 1983). It has a Km for uric acid of 10 μM and for 0_2 of 31 μM and is inhibited (competitively) by xanthine. Uricase purified from cowpea nodules is similar to the enzyme from soybean nodules except that it is inhibited by ammonia and glutamine as well as xanthine (Rainbird and Atkins 1981). The latter authors pointed out that the Km of uricase for 0_2 is very high and that this is consistent with the location of the enzyme in the uninfected rather than the bacteroid containing cells. It has been shown that uricase biosynthesis in soybean callus tissue is induced by low 0_2 concentrations with the highest levels being seen as the 0_2 concentration is lowered to 3%-4% (Larsen and Jochimsen 1986). These authors also concluded that uricase biosynthesis is independent of N_2 fixation, since nodules formed by ineffective strains of Rhizobium still synthesised normal levels of uricase. However, uricase activity of nodules formed by effective Rhizobium strains was very low in the latter study.

Phosphoribosyl pyrophosphate amidotransferase (PRAT), which catalyses the first committed step of purine biosynthesis, has been

purified from soybean nodules and is inhibited by IMP, GMP, and XMP, end products of the purine biosynthetic pathway (Reynolds et al. 1984). IMP dehydrogenase which functions at the branch point between the funneling of purines into ureide biosynthesis or purine nucleotide synthesis has been purified from cowpea nodules (Atkins et al. 1985). The activity of this enzyme is not affected by allantoin, allantoic acid, uric acid, inosine, xanthosine or XMP but is inhibited by AMP, GMP, and NADH. Whilst purine biosynthesis occurs in the plastids, IMP dehydrogenase is located in the cytosol and therefore IMP is probably the intermediate which passes between the plastids and the cytosol. If XDH is located in the uninfected cells as Nguyen et al. (1986) propose and IMP dehydrogenase is located predominantly in the infected cells (Atkins et al. 1985), then XMP is probably the intermediate which passes from the infected cells to the uninfected cells of the nodule.

1.5 Ureide Utilization in the Leaves of Soybean.

The alternative pathways of ureide utilization in the leaves of soybean, as summarized by Schubert (1986), are shown in Fig. 1.2. Until recently, little was known about these pathways except that allantoinase which catalyses the conversion of allantoin to allantoic acid in the nodules catalyses the same reaction in shoot tissues. Abundant activity of this enzyme is found in soybean leaves and fruits and accumulation of allantoate in these organs suggests that allantoate hydrolysis is the rate limiting step in ureide utilization (Thomas and Schrader 1981 a).

There have been two contradictory reports regarding the pathway of allantoate degradation. The first favoured the involvement of allantoate amidinohydrolase (=allantoicase) which catalyses the hydrolysis of allantoate to yield ureidoglycolate and urea (Shelp and Ireland 1985). A later report, however, refuted this and showed that allantoate amidohydrolase, which catalyses the formation of ureidoglycine, ammonia and CO_2 from allantoate, is the enzyme involved in ureide degradation in soybean (Winkler et al. 1985).

1.6 Carbon Metabolism in the Plant Fraction of Soybean Nodules.

The dependence of N_2 fixation on currently supplied photosynthate is now firmly established (Lawrie and Wheeler 1975, Antoniw and Sprent 1978, Reibach and Streeter 1983, Gordon et al. 1985, Kouchi et al. 1985) and the form in which photosynthate is translocated to legume nodules is as sucrose (Bach et al. 1958). The main enzymes involved in the initial degradation of sucrose in soybean nodules are alkaline invertase and sucrose synthase (Morell and Copeland 1984, 1985, Copeland and Morell 1985). These enzymes catalyse the cleavage of sucrose to yield fructose and glucose. Hexokinase and fructokinase then catalyse the phosphorylation of glucose and fructose, respectively. Alkaline invertase is the main enzyme involved in sucrose breakdown in developing soybean nodules and fructokinase predominates over hexokinase. Alkaline invertase activity is regulated mainly by the availability of sucrose and fructokinase by the ATP/ADP ratio. The further metabolism of sucrose presumably proceeds via glycolysis and the TCA cycle although this has not as yet been investigated in soybean nodules.

Another important carbon metabolising enzyme in soybean nodules is PEP carboxylase. This enzyme catalyses the refixation of respired CO_2 to yield oxaloacetate and is thereby involved in the synthesis of (1) carbon skeletons for the assimilation of fixed nitrogen, (2) respiratory substrates for the bacteroids and (3) carboxylic acids to balance excess cation charge (see Gadal 1983).

In soybean, which synthesises ureides as the major products of N_2 fixation, the importance of CO_2 fixation in providing carbon skeletons for the assimilation of fixed nitrogen, is not so great as it is in alfalfa, for example, which synthesises amides (Vance et al. 1985). Treatments which inhibit N_2 assimilation but not nitrogenase activity, such as $He:O_2$, acetylene and $Ar:O_2$, have little effect on CO_2 fixation in soybean nodules (Laing et al. 1979, Coker and Schubert 1981, King et al. 1986). Conversely, treatments, which inactivate nitrogenase, such as supraoptimal O_2, markedly reduce CO_2 fixation (Laing et al. 1979, King et al. 1986).

The involvement of carboxylic acids (particularly malate) in balancing excess cation charge in the roots was first demonstrated by Israel and Jackson (1982). These authors showed that soybean plants dependent solely on N_2 as a nitrogen source take up an excess of cations over anions and they proposed that this stimulates malate synthesis in the roots.

1.7 Nodulins.

The term "nodulin" refers to a class of proteins, encoded by the plant genome, which are only found in nodules and not in the supporting root (Legocki and Verma 1980). Proteins which can be loosely classified as nodulins, by virtue of the fact that their enzymic activities are either unique to or amplified in the plant fraction of nodules (as compared with roots), include GS, GOGAT, XDH, uricase, allantoinase, invertase, phosphofructokinase, PEP carboxylase (see Verma et al. 1983) and choline kinase II (Mellor et al. 1986). In many cases, nodule specific isozymes are involved. The conclusive classification of nodule proteins as nodulins requires not only that they are unique to nodules but also that they can be demonstrated to be encoded by plant derived mRNA. Proteins which satisfy these criteria are leghaemoglobin (Auger et al. 1979), uricase (Legocki and Verma 1979, Verma et al. 1983), glutamine synthetase (Cullimore et al. 1982, Hirel et al. 1986, in press) and sucrose synthase (Thummler et al. 1986, in press). In addition, in soybean nodules, there are three proteins with molecular weights of 24Kd, 27Kd and 40Kd which have been conclusively classified as nodulins but have not been assigned a function (Fuller et al. 1983, Fuller and Verma 1984). The mRNAs encoding these nodulins are first detectable three to five days after inoculation of the roots with Rhizobium and they accumulate rapidly when nodules first become distinct from the root tissue. The accumulation of these nodulin mRNAs coincides with the accumulation of leghaemoglobin mRNA and precedes the onset of N_2 fixation. Recently, nodulin-24 has been localized in the peribacteroid membrane along with several other nodulins (Fortin et al. 1985, Katinkas and Verma 1985). It is not unlikely that some of these

unidentified nodulins will be found to be some of the "loosely classified nodulins" (see above).

Attempts to identify nodulins have focused on the use of symbiotically defective Rhizobium mutants. For example, in soybean nodules induced by Bradyrhizobium japonicum strain SM5, an ineffective strain which produces an altered subunit of nitrogenase, normal levels of all of the nodulin mRNAs are found. On the other hand, in soybean nodules induced by the ineffective strain 61A24, the nodulin mRNAs are still present but at lower levels (Fuller and Verma 1984). In separate studies it was shown that soybean nodules induced by ineffective strains of Bradyrhizobium japonicum have lower levels of GS and GOGAT activity in the nodule cytoplasm (Duke and Ham 1976, Sen and Schulman 1980, Werner et al. 1980). However, no attempt was made to correlate these reductions with changes in the levels of nodulin mRNAs.

Other perturbations of the symbiotic system which may aid the identification of nodulin gene products include the use of plant-conditioned symbiotically ineffective mutants, nitrate treatment and exposure to an $Ar:0_2$ (essentially N_2-free) atmosphere. The latter treatment leads to a marked reduction in the level of GS, GOGAT, purine biosynthetic, XDH, uricase and allantoinase activities in cowpea nodules (Atkins et al. 1984 a, b). Similarly, ineffective mutants of alfalfa have lower levels of GS and GOGAT activity (Groat and Vance 1982). Again, neither of these studies attempted to correlate reductions in enzyme activity with reductions in the levels of nodulin mRNAs. To date

there have been no reports on the effect of nitrate treatment on the enzymic activities of the "loosely classified" nodulins. Neither have there been any attempts made to correlate these effects of nitrate treatment with any effects nitrate may have on nodulin mRNA levels.

1.8 Statement of Approach.

This study set out to identify plant derived nodule proteins which were associated with the inhibitory effects of nitrate on nodulation and nitrogenase activity. The aim was to make a contribution to the understanding of the mechanism of nitrate inhibition. The approach was two-fold. Firstly, the effect of nitrate treatment on enzymes involved in N_2 assimilation and carbon metabolism in the nodules of soybean cv. Bragg was examined. Secondly, soybean genotypes with reduced susceptibility to the inhibitory effects of nitrate, were compared with the nitrate susceptible cultivar Bragg. This second approach included an analysis of nts382, a supernodulating, nitrate-tolerant mutant derivative of soybean cv. Bragg, (Carroll et al. 1985 a, b). Comparative analyses of enzyme activities were coupled with analyses of bacteroid-free nodule extracts using polyacrylamide gel electrophoresis.

Figure 1.1 Pathway and Location of Ammonia Assimilation and Ureide Biosynthesis in the Nodules of Ureide-Producing Legumes, as proposed by Schubert (1986). It should be noted that this figure is in some respects hypothetical. For example, in cowpea at least, the enzyme which oxidises IMP to XMP (IMP dehydrogenase) is located in the cytosol and not in the plastids as shown in the figure (Atkins et al. 1985). Furthermore, IMP and not XMP is probably the intermediate which passes from the plastids to the cytosol. There is still some uncertainty as to whether XDH, which catalyses the oxidation of xanthine to uric acid, is located in the infected (Triplett 1985) or the uninfected (Nguyen et al. 1986) cells of the nodule.

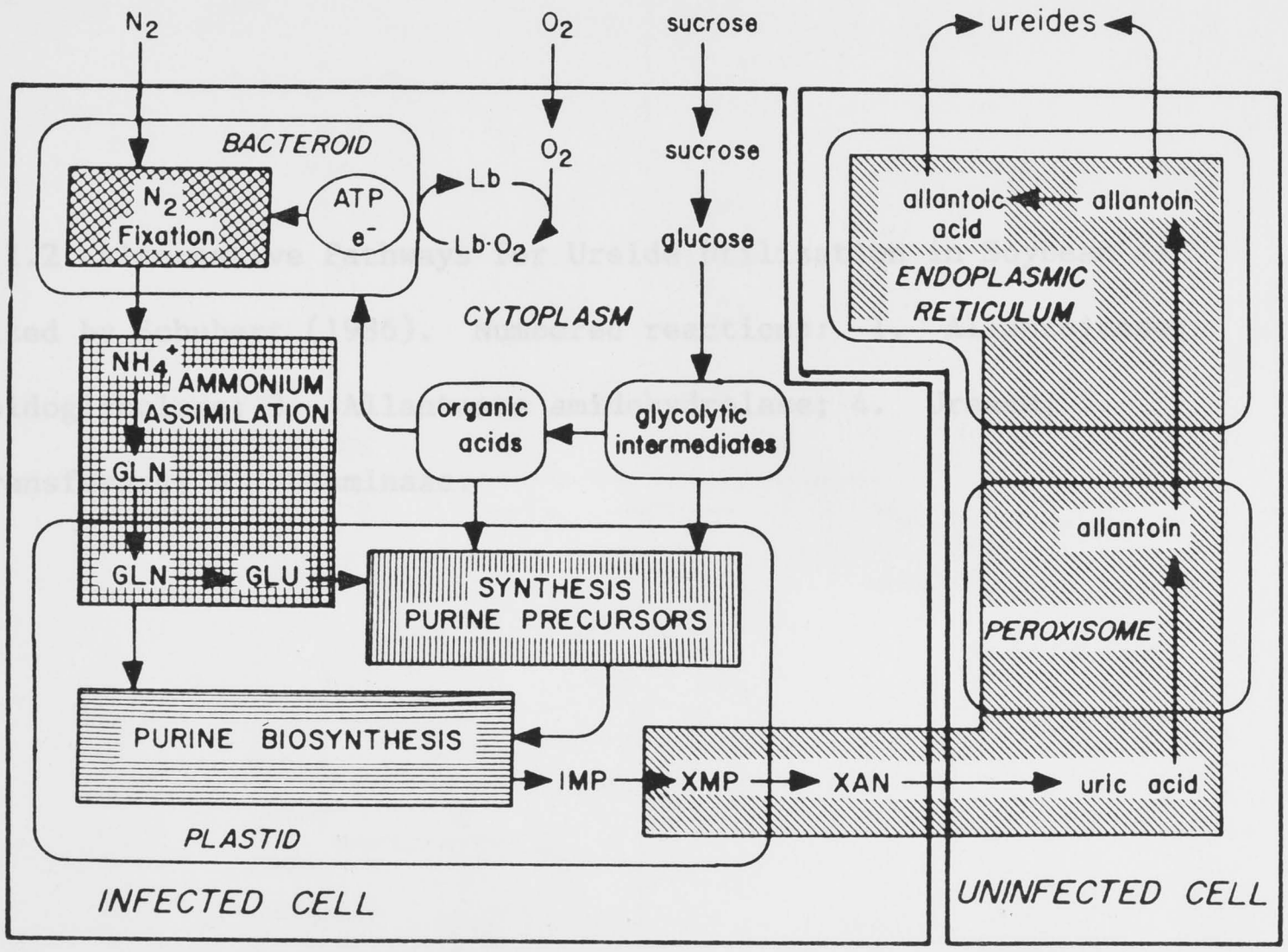
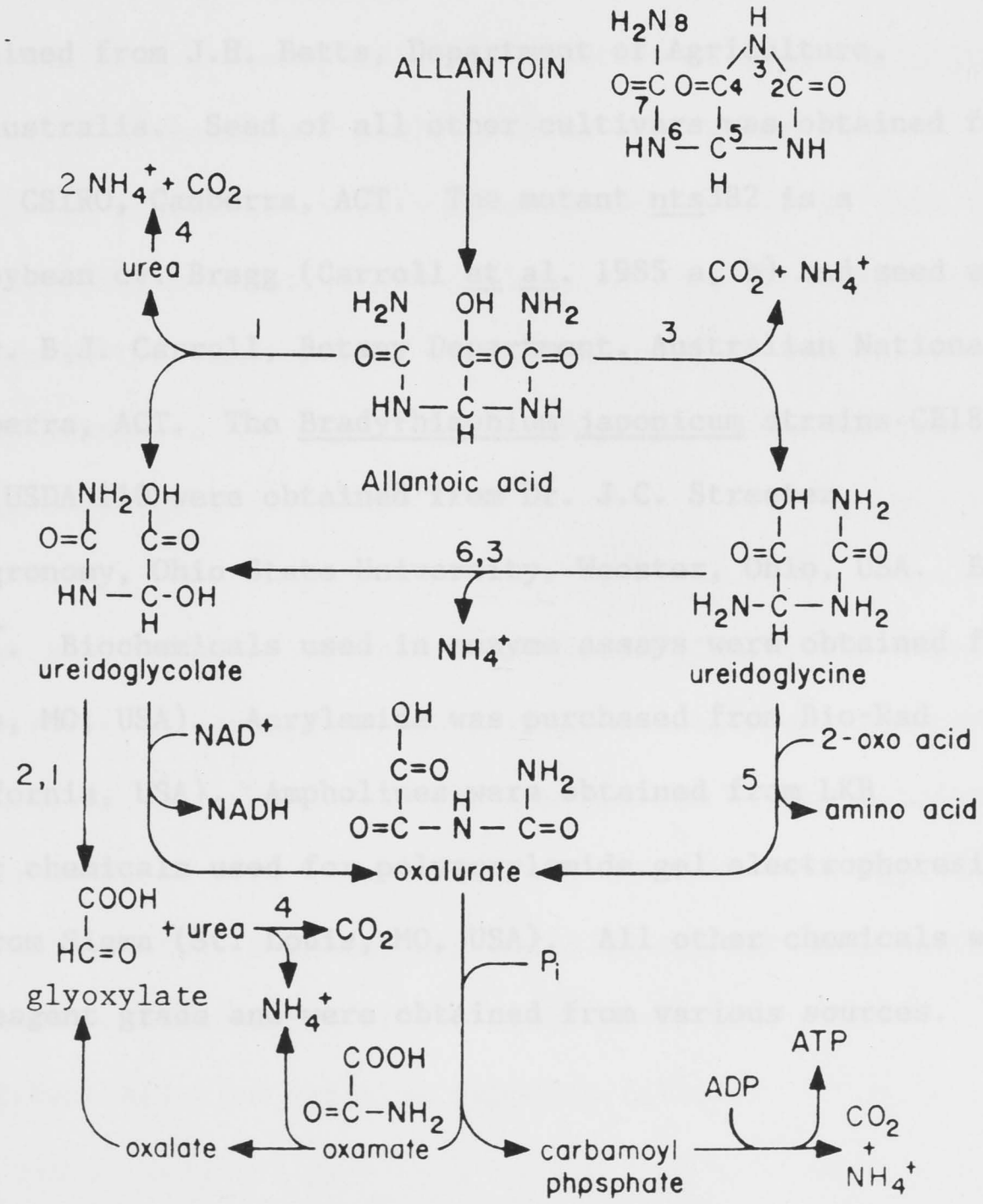

N2
O2
sucrose
ureides
BACTEROID
N2 Fixation
ATP
e-
Lb
Lb·O2
O2
sucrose
glucose
CYTOPLASM
NH4+ AMMONIUM ASSIMILATION
GLN
GLN → GLU
organic acids
glycolytic intermediates
SYNTHESIS PURINE PRECURSORS
PURINE BIOSYNTHESIS
IMP
XMP
XAN
uric acid
PLASTID
INFECTED CELL
allantoic acid
allantoin
ENDOPLASMIC RETICULUM
allantoin
PEROXISOME
UNINFECTED CELL

Figure 1.2 Alternative Pathways for Ureide Utilization in Soybean as summarized by Schubert (1986). Numbered reactions: 1. Allantoicase; 2. Ureidoglycolase; 3. Allantoate amidohydrolase; 4. Urease; 5. Aminotransferase; 6. Deaminase.


ALLANTOIN
2 NH4+ + CO2
4
urea
1
3
CO2 + NH4+
Allantoic acid
6,3
NH4+
ureidoglycolate
ureidoglycine
NAD+
NADH
2,1
5
2-oxo acid
amino acid
oxalurate
COOH
HC=O
+ urea
4
CO2
glyoxylate
NH4+
Pi
COOH
O=C-NH2
oxalate
oxamate
carbamoyl
phosphate
ADP
ATP
CO2
+
NH4+

CHAPTER 2

Material and Methods

2.1 Materials

Seed of the soybean (Glycine max L. Merr.) cultivars Bragg, Leslie and Lee was obtained from J.H. Betts, Department of Agriculture, Tamworth, NSW, Australia. Seed of all other cultivars was obtained from Dr. A.H. Gibson, CSIRO, Canberra, ACT. The mutant nts382 is a derivative of soybean cv. Bragg (Carroll et al. 1985 a, b) and seed was obtained from Dr. B.J. Carroll, Botany Department, Australian National University, Canberra, ACT. The Bradyrhizobium japonicum strains CB1809 (=USDA 136) and USDA 110 were obtained from Dr. J.C. Streeter, Department of Agronomy, Ohio State University, Wooster, Ohio, USA. Both strains are Hup^+. Biochemicals used in enzyme assays were obtained from Sigma (St. Louis, MO, USA). Acrylamide was purchased from Bio-Rad (Richmond, California, USA). Ampholines were obtained from LKB (Sweden). Other chemicals used for polyacrylamide gel electrophoresis were obtained from Sigma (St. Louis, MO, USA). All other chemicals were analytical or reagent grade and were obtained from various sources.

2.2 Plant Culture

2.2.1 Standard Plant Culture

Soybean seeds were planted in 8 l pots of either sand, vermiculite or a 1:1 mixture of sand:vermiculite (4-10 seeds per pot). The pots were then inoculated with a slurry of a peat culture of Bradyrhizobium japonicum (usually > 10^9 cells per pot). Plants were grown in a naturally illuminated glasshouse with supplementary heating so that the temperature did not fall below 15°C and cooling so that the temperature did not rise above 30°C. Considerable variation in plant growth rate occurred from one season to the next. This was probably due to seasonal variation in light intensity.

Prior to the imposition of nitrate treatments, plants were supplied three-times weekly with a nitrogen-free nutrient solution adapted from Herridge (1977) and daily with water. The composition of the nutrient solution is shown in Table 2.1. When nitrate treatments were applied, solid KCl (control) or KNO_3 (treated) was added to the nutrient solution to give the required concentration of the anion (5-10 mM). The supplemented nutrient solution was then supplied daily.

2.2.2 Hydroponic Plant Culture

Plants were grown in LB cabinets as described previously (Harper and Gibson 1984) except that (i) they were inoculated with

Bradyrhizobium japonicum strain CB1809 3 d after setting to germinate and 3 d before transfer to hydroponics pots, (ii) 1 mM KNO_3 was supplied 18 DAP and maintained at that concentration until the nodulated roots were assayed for nitrogenase (acetylene reduction) activity 2 d later. The plants were grown 4 per pot and the pots were arranged in randomized blocks (4 pots per treatment). The growth conditions were 28°C/14 h light and 19°C/10 h dark. The light intensity was 600 µE. m^{-2}. s^{-1}.

2.3 Nitrogenase Assays

2.3.1 Standard Acetylene Reduction Assay

Nodulated root systems which had had their shoots removed were placed in 1 l gas tight jars fitted with a rubber stopper. Acetylene (50 ml) was added by means of a syringe to give a final concentration of acetylene in air of 5% (v/v). After 20 min incubation at room temperature (20 - 25°C), 0.2 ml gas samples were removed and analysed for ethylene by FID-GC.

2.3.2 Comparative Acetylene Reduction and $^{15}N_2$ Fixation Assay

Single root systems trimmed to the well nodulated crown region were placed in 37 ml glass vials sealed with a rubber stopper. The vials were injected with either 3.0 ml N_2 (98% $^{15}N_2$) and 0.8 ml 0_2 or 4.0 ml acetylene and 0.8 ml 0_2. After 30 min incubation, samples were removed from the acetylene vials and analysed for ethylene by FID-GC and the $^{15}N_2$ vials were opened and vented. The material exposed to $^{15}N_2$ was

digested and analysed for ^{15}N (Bergersen 1980) using a Micromass 903 (V.G. Micromass, Winsford, Cheshire UK) mass spectrometer.

2.4 Bacteroid Isolation and Assay for Acetylene Reduction Activity

Bacteroids were isolated anaerobically in a modified Sorvall Omnimix blender (Sandeman and Gresshoff 1985). The isolation buffer, which was thoroughly sparged with either N_2 or argon prior to contact with the isolated bacteroids, consisted of 50 mM KH_2PO_4 (pH 7.3), 0.15 M NaCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 4% (w/v) PVP, 20 mM dithionite and 2 mM DTT. Following isolation, the bacteroids were pelleted by centrifugation at 2,000 g for 5 min and then resuspended in a modification of the isolation buffer lacking PVP, dithionite and DTT. This process was repeated twice. Aliquots (0.5 ml) of the final resuspension (2 mg protein. ml^{-1}) to be assayed for acetylene reduction activity, were then injected into 22 ml glass vials containing an atmosphere of 0.2%, 0.5%, 1.0%, 1.5% or 2.0% (v/v) O_2 in N_2 or argon and 0.5 ml of the modified isolation buffer. The O_2 in N_2 or argon atmospheres were generated by first of all replacing the air in the vials using a gas manifold designed to carry out repeated cycles of evacuation and flushing of the vials with N_2 or argon. The required volume of air was then added with a 1 ml plastic disposable syringe to give the specified concentrations of O_2. Malate (5 mM, final concentration) was added to the assay vials where indicated in Tables and Figures. The assay was initiated by the addition of acetylene (5% (v/v), final concentration) and the vials were shaken at 25°C. Gas samples were removed after 20 min and analysed for ethylene by FID-GC.

2.5 Estimation of Bacteroid Protein Content of Nodules

The bacteroid protein content of nodules was estimated using β-hydroxybutyrate dehydrogenase (β-HBDH) as a bacteroid specific marker (Wong and Evans 1971). Activity of β-HBDH in bacteroid preparations and nodule homogenates was measured by following the oxidation of NADH in the presence of acetoacetate. The reaction mixture contained, in a final volume of 1 ml, 50 µmol TES buffer (pH 7.0), 0.2 µmol KCN, 0.020 µmol NADH and 50 µl of sonicated (2 min, 300 W) bacteroids or nodule homogenate. The reaction was initiated by adding acetoacetate (5 mM, final concentration) and the decrease in optical density at 340 nm was measured with a Pye-Unicam P8800 spectrophotometer. The bacteroid protein content of nodules was calculated by dividing β-HBDH activity per g nodule fresh weight by bacteroid β-HBDH activity per mg bacteroid protein. Bacteroid protein was determined by the method of Lowry *et al.* (1951).

2.6 Collection of Xylem Sap

Xylem sap was collected, over a period of 20 min, under root pressure, into plastic tubing fitted around the top of decapitated root systems. The rooting medium was saturated with water prior to decapitation of the plants. This stimulated exudation of the sap. Little exudation occurred otherwise. The volume of sap exuded was estimated from the weight, assuming a density of 1 g/cm^3. Samples were stored frozen at -70°C for later analysis for ureides, α-amino-nitrogen and nitrate.

2.7 Preparation of Nodule and Leaf Extracts for Ureide Analysis

Nodules or leaves were homogenized in a mortar and pestle in 10 volumes of 0.25 N $HC1O_4$. The homogenate was centrifuged at 10,000 g for 15 min and the supernatant was neutralized with the required volume of KOH. The resultant $KC1O_4$ precipitate was removed by centrifugation and the supernatant retained for analysis (Polayes and Schubert 1984).

2.8 Chemical Analyses

2.8.1 Nitrate

Nitrate was determined in the xylem sap by the salicylic acid method (Cataldo et al. 1975). A 0.4 ml aliquot of 5% (w/v) salicylic acid in concentrated H_2SO_4 was added to 0.1 ml of the sample to be analysed. After standing for 20 min at room temperature (20 - 25°C), 9.5 ml of 2 N NaOH was added and the optical density was read at 410 nm. A nitrate standard curve was prepared in the range 0-1 µmol KNO_3.

2.8.2 α-Amino Nitrogen

A modification of the ninhydrin method of Yemm and Cocking (1955) was used to determine α-amino nitrogen in the xylem sap (Herridge 1984). A 0.5 ml aliquot of 16.8% (w/v) citrate in 6.4% (w/v) NaOH was added to 50 µl of the sample to be analysed, followed by 1.0 ml of the ninhydrin reagent which consisted of 0.96% (w/v) ninhydrin and 0.033% (w/v) ascorbate in 2-methoxyethanol. Following a 25 min incubation at 100°C,

3 ml of 60% (v/v) aqueous ethanol was added and the optical density was read at 570 nm. An asparagine standard curve was prepared in the range 0-1 µmol.

2.8.3 Ureides

Ureides were determined as the phenylhydrazone of glyoxylate (Vogels and van der Drift 1970). The procedure was as follows.

Step 1: A 0.1 ml aliquot of the sample to be analysed was incubated for 8 min at 100°C with 0.2 ml distilled water and 0.1 ml 0.5 N NaOH. The mixture was then cooled in ice water.

Step 2: A 0.1 ml aliquot of 0.565 N HCl was added and the reaction mixture was incubated for 4 min at 100°C. The reaction mixture was then cooled in ice water and 0.1 ml 0.4 M Na_2HPO_4 (pH 7.0) was added.

Step 3: A 0.1 ml aliquot of phenylhydrazine-HCl (100 mg. 30 ml^{-1}) was added and the reaction mixture was allowed to stand at room temperature for 5 min.

Step 4: The reaction mixture was returned to the ice water bath and 0.5 ml concentrated HCl and 0.1 ml potassium ferricyanide (500 mg. 30 ml^{-1}) was added. Colour was allowed to develop for 15 min at room temperature and then the optical density was read at 535 nm.

Steps 1-4 determined allantoin, allantoate, ureidoglycolate and glyoxylate. Steps 2-4 determined all of the above excluding allantoin. Steps 3-4 determined glyoxylate alone. Allantoin, allantoate and glyoxylate standard curves were prepared in the range 0-0.1 µmol.

2.8.4 Nitrite

A 0.4 ml aliquot of the sample to be analysed was reacted with 0.6 ml of a 1:1 mixture of 1% sulphanilamide in 3 N HCl and 0.02% N-naphthyl-ethylene-diamine HCl in distilled water (Scholl et al. 1974). After allowing 20 min for colour development, the optical density was read at 540 nm. A nitrite standard curve was prepared in the range 0-40 nmol KNO_2.

2.8.5 Haem

The method was as described by Appleby and Bergersen (1980). A suitable dilution of nodule extract was mixed with an equal volume of alkaline pyridine reagent and a few crystals of sodium dithionite. The absorbance was then measured at 556 nm against a reagent blank. The haem content was calculated as follows:

$$\text{Haem concentration (mM)} = (A_{556nm} \times 2D)/\varepsilon$$

D = the initial dilution of the nodule extract and

$$\varepsilon = 33.9 \times 10^3 \text{ l. mol}^{-1}\text{. cm}^{-1}$$

The alkaline pyridine reagent, 4.2 M pyridine in 0.2 M NaOH, was prepared as follows: 0.8 g of NaOH was dissolved in 50 ml of water and

allowed to cool; then 33.8 ml of pyridine was added and the volume was made up to 100 ml with water.

2.8.6 Protein

Protein was determined either by the method of Lowry et al. (1951) or by the method of Bradford (1976) using BSA as the standard.

2.9 Enzyme Assays

2.9.1 Glutamine Synthetase

Nodules (0.3-0.5 g) were homogenised in a mortar and pestle in 3.0-5.0 ml of an extraction medium containing 100 mM MES buffer (pH 6.8), 100 mM sucrose, 2% (v/v) mercaptoethanol and 15% (v/v) ethylene glycol (Groat and Vance 1981). The homogenate was filtered through "Miracloth" and centrifuged at 30,000 g for 30 min. The supernatant was retained for enzyme assays. Glutamine synthetase (GS) activity was assayed by a modification of the γ-glutamyl transferase assay of Cullimore and Sims (1980). The assay medium consisted of 100 μmol MES buffer (pH 6.4), 100 μmol glutamine, 60 μmol hydroxylamine, 0.5 μmol ADP, 1.0 μmol $MnCl_2.4H_20$, 20 μmol sodium arsenate and 0.1 ml nodule extract (approximately 5.0 mg protein. ml^{-1}) in a final volume of 0.9 ml. The reaction was initiated by addition of the extract and terminated after 20 min incubation at 30°C by addition of 1.0 ml of a "stop mix" consisting of 4% (w/v) TCA and 3.2% (w/v) ferric chloride in 0.5 N HCl. The optical density at 500 nm was read following

centrifugation to remove acid precipitable compounds. Reactions "stopped" at time zero were used to correct for non-specific absorption at 500 nm. A glutamyl hydroxamate standard curve was prepared in the range 0 - 5 μmol.

2.9.2 Glutamine Oxoglutarate Aminotransferase

Nodule extracts were prepared as for the GS assay and glutamine oxoglutarate aminotransferase (GOGAT) activity was assayed by the method of Groat and Vance (1981). The assay medium consisted of 100 μmol KH_2PO_4 (pH 7.5), 0.1% (v/v) mercaptoethanol, 100 nmol NADH, 2.5 μmol α-ketoglutarate, 10 μmol glutamine, 1 μmol amino-oxyacetate and 0.2 ml nodule extract (approximately 5.0 mg protein. ml^{-1}) in a final volume of 1.0 ml. The reaction was initiated by addition of the extract and the decrease in optical density due to glutamine dependent NADH oxidation was monitored at 340 nm. Corrections were made for glutamine independent NADH oxidation by running assays in the absence of glutamine.

2.9.3 Xanthine Dehydrogenase

The method was essentially that of Schubert (1981). Nodules (0.3-0.5 g) were homogenised in a mortar and pestle in 3.0-5.0 ml of an extraction medium containing 25 mM TES buffer (pH 7.5), 0.3 M sorbitol, 1 mM DTE and insoluble PVP (0.1 g. g^{-1} nodule fresh weight). The homogenate was filtered through "Miracloth" and centrifuged at 75,000 g. The supernatant was retained for enzyme assays. Xanthine dehydrogenase

(XDH) activity was assayed at room temperature by monitoring the increase in optical density at 340 nm due to NAD reduction. The assay medium consisted of 75 µmol TES (pH 8.4), 6.25 µmol NAD, 0.25 µmol xanthine and 0.25 ml of nodule extract (approximately 5 mg protein. ml^{-1}) in a final volume of 1.5 ml. The reaction was initiated by addition of the nodule extract. Corrections were made for xanthine-independent NAD reduction by performing assays in the absence of xanthine.

2.9.4 Uricase

Nodule extracts were prepared as for the XDH assay. Uricase activity was assayed at room temperature by monitoring the decrease in optical density at 292 nm due to the disappearance of uric acid. The assay medium consisted of 0.75 µmol CHES buffer (pH 10), 0.15 µmol uric acid and 0.1 ml nodule extract (approximately 5.0 mg protein. ml^{-1}) in a final volume of 1.25 ml (Schubert 1981). The reaction was initiated by addition of the nodule extract. Results were corrected for non-enzymic uric acid degradation by carrying out assays in the absence of the nodule extract.

2.9.5 Allantoinase

2.9.5.1 <u>In Vitro</u> Activity in Nodules

The method was a modification of that of Schubert (1981). Nodule extracts were prepared as for the XDH assay except that DTE was excluded

from the extraction medium. Allantoinase activity was assayed by determining the rate of formation of allantoate from allantoin. The assay medium consisted of 1.0 ml 25 mM allantoin, 1.0 ml 0.1 M TES buffer (pH 8.1) and 0.1 ml nodule extract (approximately 5.0 mg protein. ml^{-1}). The reaction was initiated by addition of the nodule extract. After 20 min incubation at 37°C, 0.5 ml of 0.15 N HCl was added and the assay tubes were incubated for a further 4 min at 100°C. Following cooling in an ice-water bath, 0.5 ml phenylhydrazine-HCl (3 mg. ml^{-1}) was added and the reaction tubes were allowed to stand at room temperature for 10 min. Cooling prior to the addition of phenylhydrazine-HCl was the most critical factor in preventing the formation of artefacts which interfered with allantoate determination. The reaction tubes were returned to the ice water bath prior to the addition of 1.2 ml precooled (4°C) concentrated HCl and 0.5 ml potassium ferricyanide (16 mg. ml^{-1}). Colour was allowed to develop for 15 min at room temperature and the optical density was then read at 520 nm. A standard curve was prepared in the range 0-0.1 µmol allantoate. Corrections were made for allantoate present at time zero by adding 0.15 N HCl to the assay medium prior to the addition of the nodule extract.

2.9.5.2 In Vivo Activity in Leaves

Allantoinase activity was assayed by determining the rate of formation of allantoate from allantoin. The method was a modification of that described by Shelp and Ireland (1985). Twelve leaf disks (1 cm diameter) were collected into 4.0 ml of assay medium standing on ice and containing 100 mM KH_2PO_4 (pH 7.5), 1% propanol, 0.1% BSA, 0.01% Triton

X-100 and 100 mM allantoin (saturated solution). The reaction was initiated by placing the assay tubes in a shaking water bath at 30°C. After 2-3 h, 1.0 ml aliquots were removed and assayed for allantoate as described in section 2.9.5.1. A correction was made for non-enzymic formation of allantoate by running assays in the absence of leaf disks. Allantoate present in the assay medium at time zero was corrected for by assaying aliquots prior to transfer of the assay tubes to the 30°C water bath.

2.9.6 Allantoate Hydrolysis

The <u>in vivo</u> rate of allantoate hydrolysis in leaves was assayed by determining the rate of glyoxylate production from allantoin. The method was a modification of that described by Shelp and Ireland (1985). The incubation procedure was as described in section 2.9.5.2. After 3 h of incubation, 1.0 ml aliquots of the incubation medium were removed into eppendorf tubes on ice. The eppendorf tubes were centrifuged to remove suspended allantoin and 0.5 ml aliquots were taken and added to 0.1 ml phenylhydrazine-HCl (3.33 mg. ml^{-1}) in reaction tubes on ice. After 5 min incubation at room temperature, the reaction tubes were placed in an ice-water bath and 0.5 ml precooled (4°C) concentrated HCl and 0.1 ml potassium ferricyanide (16.67 mg. ml^{-1}) were added. Colour was allowed to develop at room temperature for 15 min and then the optical density was read at 535 nm. A correction was made for non-enzymic glyoxylate formation by performing assays in the absence of leaf disks. Glyoxylate present in the assay medium at time zero was

corrected for by assaying aliquots prior to the transfer of the assay tubes to the 30°C water bath..

2.9.7 Invertase

Invertase activity was assayed _in vitro_ as previously described (Morell and Copeland 1984). Nodules (0.4 g) were homogenized in 1 ml of 50 mM KH_2PO_4 (pH 8), 1 mM EDTA in a mortar and pestle. The homogenate was filtered through "Miracloth" and the filtrate centrifuged at 30,000 g for 10 min. The supernatant was retained for the enzyme assay. The assay mixture consisted of 0.1 ml 50 mM KH_2PO_4 (pH 8), 0.1 ml 250 mM sucrose and 50 µl of the nodule extract. Incubations were carried out for 20 min at 30°C. The reaction was stopped by heating for 2 min in a boiling water bath and the precipitate removed by centrifugation. Glucose was determined in the supernatant by incubating 10 µl with 1.5 ml of the glucose SVR reagent (Calbiochem. Behring Australia, Kingsgrove, NSW, Australia).

2.9.8 Fructokinase

Fructokinase activity was assayed _in vitro_ as previously described (Copeland _et al_. 1978). Nodule extracts were prepared as for the invertase assay (see above). The reaction mixture contained, in a total volume of 3 ml, 75 µmol Tris-HCl (pH 8), 0.6 µmol fructose, 3 µmol ATP, 4.5 µmol $MgCl_2$, 1 µmol NADP, 72 units P-glucose isomerase (Sigma, St. Louis, MO, USA), 20 units glucose-6-P dehydrogenase (Sigma, St. Louis, MO, USA) and 50 µl of the nodule extract. The reaction was followed by

monitoring the increase in absorbance at 340 nm due to the reduction of NADP to NADPH. A correction was made for fructose independent NADPH production.

2.9.9 PEP Carboxylase

Nodule extracts were prepared and PEP carboxylase was assayed as described by Deroche et al. (1983). Nodules (1.0 g) were homogenised in a mortar and pestle in 2.0 ml of an extraction medium containing 50 mM Tris-HCl (pH 7.8), 10 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 10% (v/v) glycerol, BSA (1 mg. ml^{-1}) and PVP (1 g. g^{-1} nodule fresh weight). The homogenate was filtered through "Miracloth" and centrifuged at 48,000 g for 10 min. The supernatant was retained for the enzyme assay. PEP carboxylase activity was assayed in a medium consisting of 100 µmol Tris HCl (pH 8.0), 10 µmol $MgCl_2$, 0.3 µmol NADH, 5 µmol $NaHCO_3$, 0.05 units malate dehydrogenase (obtained from Sigma, St. Louis, MO, USA), 3 µmol PEP and 100 µl of nodule extract in a final volume of 2.7 ml. Activity was determined by monitoring PEP dependent NADH oxidation at 340 nm. Assays performed in the absence of PEP were carried out to correct for NADH oxidation not dependent on PEP.

2.9.10 Nitrate Reductase

Nitrate reductase activity was measured by determining the rate of nitrite production from nitrate. The method was a modification of that of Carroll (1980). Four leaf disks (1 cm diameter), taken from the youngest fully expanded trifoliate, were collected into 5 ml of ice-cold

assay medium containing 50 mM KNO_3 (plus nitrate assay only), 100 mM KH_2PO_4 (pH 7.5), 1% propanol, 0.1% BSA, chloramphenicol (5 mg. l^{-1}) and 0.01% Triton X-100. The reaction was initiated by placing the assay tubes in a 30°C water bath. After 40 min incubation, 0.4 ml aliquots were removed for nitrite determination (see section 2.8.4). A correction was made for nitrite present at time zero by assaying aliquots of the assay medium prior to transfer to the 30°C water bath. Once the assay was complete, the leaf disks were dried at 80°C for 24 h to enable expression of nitrate reductase activity on a tissue dry weight basis.

2.10 Polyacrylamide Gel Electrophoresis (PAGE)

2.10.1 One Dimensional SDS-PAGE

2.10.1.1 Gel Preparation

The method for preparing discontinuous, SDS-polyacrylamide slab gels was based on that of Laemmli and Favre (1973). The dimensions of the gels were 14 cm x 14 cm x 1.5 mm. The resolving gel consisted of 9.92% (w/v) acrylamide, 0.27% (w/v) bis-acrylamide, 0.375 M Tris-base (pH 8.8), 0.1% (w/v) SDS, 0.0625% (v/v) TEMED and 0.0125% (w/v) ammonium persulphate. The stacking gel consisted of 3.68% (w/v) acrylamide, 0.1% (w/v) bis-acrylamide, 0.125 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.1% (v/v) TEMED and 0.03% (w/v) ammonium persulphate. The composition of the reservoir buffer was as follows: 0.025 M Tris-base (pH 8.3), 0.2 M glycine and 0.1% (w/v) SDS.

2.10.1.2 Preparation of Nodule Extracts

Nodules extracts were prepared by homogenising 0.5 g nodules in 5 ml of 50 mM Tris-HCl (pH 7.5), 5 mM cysteine, 1 mM PMSF and insoluble PVP (0.1 g. g^{-1} nodule fresh weight) in a mortar and pestle. The homogenate was then centrifuged at 12,000 g for 10 min and the supernatant was retained.

2.10.1.3 Sample Preparation and Running of Gels

Samples were prepared for electrophoresis by diluting an aliquot of the nodule extract with an equal volume of SDS-sample buffer (0.0625 M Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2.3% (w/v) SDS and several grains of bromophenol blue). Following this, 10% (v/v) 2-mercaptoethanol was added and the sample was heated in a waterbath at 100°C for 5 min. The sample (10-50 µl, 20-100 µg protein) was then loaded onto the gel. An initial current of 20 mA was applied across the gel until the sample had passed through the stacking gel. The current was then increased to 50 mA once the sample had entered the resolving gel. The run was terminated when the bromophenol blue dye front reached the bottom of the gel (approximately 3-4 hours). The gel was then silver stained (see section 2.10.3).

2.10.2 Two Dimensional PAGE

2.10.2.1 Gel Preparation

The first dimension isoelectric focusing (IEF) gels were prepared in glass tubes with an internal diameter of 1.5 mm and a length of 14 cm. The method was essentially that of O'Farrell (1975). The second dimension SDS gel was prepared as described in section 2.10.1.1 except that the stacking gel was shorter and the well-forming comb was omitted.

2.10.2.2 Sample Preparation and Running of Gels

Nodule extracts were prepared as described for one-dimensional PAGE (see section 2.10.1.2). Samples for electrophoresis were prepared by adding 0.28 ml of the nodule extract to 0.57 g of urea, 0.20 ml 10% (v/v) NP-40, 40 µl 40% (w/v) Ampholines pH 5-7, 10 µl 40% (w/v) Ampholines pH 3.5-10 and 50 µl of 2-mercaptoethanol to give a final volume of 1.0 ml and a urea concentration of 9.5 M. Once the urea was dissolved, 10-50 µl of the sample (20-100 µg protein. ml^{-1}) was loaded onto the first-dimension IEF gel which had been pre-run at 200 V for 15 min, 300 V for 30 min and 400 V for 30 min. The proteins in the sample were then focused at 400 V for 22 h and 800 V for 1 h. The method for running the first dimension IEF gels was essentially that of O'Farrell (1975) except that the upper reservoir of the electrophoresis tank contained 100 mM NaOH and the lower reservoir 50 mM H_3PO_4. Once the run was complete, the IEF gel was exuded from its glass tube under air pressure using a syringe with plastic tubing attached. The gel was then

equilibrated in half-strength SDS-sample buffer for 30 min-2 h prior to loading onto the second dimension SDS gel. The two gels were sealed together with 1% agarose in half strength SDS-sample buffer. The second dimension was then run as described in section 2.10.1.3.

2.10.3 Staining of Gels

2.10.3.1 Coomassie Blue Stain

Gels were stained without shaking for at least 4 h in 0.25% (w/v) Coomassie Blue, 45% (v/v) methanol and 10% (v/v) glacial acetic acid and subsequently destained (with shaking) in four changes (1 h each) of 25% (v/v) ethanol, 10% (v/v) glacial acetic acid.

2.10.3.2 Silver Stain

The method was based on that of Sammons et al. (1981). The procedure was essentially as for the Coomassie Blue stain except that Coomassie Blue was omitted from the initial fixative and two extra destaining steps (1 h each) in 10% (v/v) ethanol, 0.5% (v/v) glacial acetic acid were added. Following destaining, the gels were incubated (with shaking) in 1.9 g. l^{-1} $AgNO_3$ for 2 h and then rinsed briefly in distilled water. They were then incubated (with shaking) in the developer which consisted of 0.75% (v/v) formaldehyde in 0.75 N NaOH. Development was allowed to proceed until protein bands or spots were visible and then it was stopped by exchanging the developer for 5% (v/v) glacial acetic acid.

Table 2.1 Herridge's Nutrient Solution

Chemical[a]	Final concentration (mg. l^{-1})
KH_2PO_4[b]	17.0
K_2HPO_4	21.8
KCl[b]	18.7
$MgSO_4.7H_2O$[b]	123.3
$CaCl_2$	27.7
ferric monosodium salt of EDTA[c]	8.7
H_3BO_3[d]	71.5×10^{-2}
$MnCl_2.4H_2O$[d]	45.3×10^{-2}
$ZnCl_2$[d]	2.8×10^{-2}
$CuCl_2.2H_2O$[d]	1.3×10^{-2}
$NaMoO_4.2H_2O$[d]	0.6×10^{-2}

[a] chemicals were prepared as stock solutions and diluted in tap water

[b] administered from 1M stock solutions

[c] administered from a 4,000 times stock solution

[d] these chemicals were collectively prepared in a 4,000 times stock solution

CHAPTER 3

The Effect of Nitrate Treatment on N_2 Fixation: A Comparison of Assays

3.1 Introduction

Nitrogenase, the enzyme which catalyses the reduction of N_2 to ammonia, also catalyses the reduction of H and a number of triple bonded compounds including cyanide and acetylene (Dilworth 1966, Schollhorn and Burris 1966). The nitrogenase catalysed reduction of acetylene to ethylene is used extensively as an assay of N_2 fixation. Recently, however, it has been shown to underestimate N_2 fixation, in some cases, since replacement of N_2 with acetylene results in a decline in nitrogenase activity and the associated respiration (Minchin *et al.* 1983). Argon has the same effect as acetylene indicating that it is the cessation of ammonia production, rather than the presence of acetylene *per se* or the production of ethylene, which induces the decline in nitrogenase activity (Minchin *et al.* 1983). It is postulated that the decline in respiration is causal rather than being a result of diminished nitrogenase activity and is due to an increase in O_2-diffusion resistance in the nodule (Sheehy *et al.* 1983). This effect has been observed with white clover, pea, lucerne and soybean cv. Fiskeby V but not with sainfoin or soybean cv. Clarke (Minchin *et al.* 1983).

In view of the variability between species, and more importantly between soybean cultivars, this chapter examines the acetylene reduction assay and compares it with other methods for assaying N_2 fixation. N_2 fixation was estimated directly using acetylene reduction and $^{15}N_2$ fixation and indirectly from the ureide content of xylem sap. The latter method depends on the observation that ureide production is virtually uniquely coupled to N_2 fixation in soybean (see Schubert and Boland 1984).

3.2 Results and Discussion

3.2.1 Acetylene Reduction Activity and $^{15}N_2$ Fixation

The effect of nitrate treatment on acetylene reduction activity (ARA) and $^{15}N_2$ fixation in soybean cv. Bragg is illustrated in Table 3.1. ARA was inhibited by 44% and 60% after 2 d and 6 d, respectively, of nitrate treatment. Similar levels of nitrate induced inhibition (47% after 2 d and 58% after 6 d) were observed when N_2 fixation was assayed using $^{15}N_2$. This similarity indicates that ARA is a reliable measure of the effect of nitrate treatment on nitrogenase activity of soybean cv. Bragg, regardless of whether or not it underestimates N_2 fixation.

3.2.2 Xylem Sap Ureide Content

N_2 fixation was also estimated from the ureide concentration in the xylem sap. This was decreased by 66% and 46% after 2 d and 6 d,

respectively, of nitrate treatment (Table 3.2). Thus, the effect of nitrate treatment on this parameter was similar to the effect on ARA and $^{15}N_2$ fixation (Table 3.1). The small discrepancy between the methods can probably be explained by the fact that xylem sap ureide concentration reflects whole plant N_2 fixation whereas ARA and $^{15}N_2$ fixation were expressed on a nodule fresh weight basis. It was not possible to calculate whole plant ARA and $^{15}N_2$ fixation in this experiment because only the crown region of the roots was assayed (see Chapter 2).

Another explanation for the discrepancy between the methods may be variations in the xylem sap exudation rate leading to changes in the ureide concentration. Variations in the xylem sap exudation rate, both diurnally and over the exudate collection period, have been shown to be inversely correlated with variations in the total nitrogen content of the exudate (McClure and Israel 1979, McClure et al. 1980). To overcome problems associated with this, these authors proposed expressing ureides as a percentage of total nitrogen in the xylem sap. This expression, known as the relative abundance of ureides, has been shown to be positively correlated with the contribution of N_2 fixation to total plant nitrogen for a range of applied nitrate concentrations (Herridge 1982, 1984).

Assuming that the sum of ureides, nitrate and α-amino nitrogen accounts for virtually all of the nitrogen in the xylem sap (McClure and Israel 1979), the data in Table 3.2 were used to calculate the relative abundance of ureides. The values for control plants were 79% and 61%, after 2 d and 6 d, respectively, of nitrate treatment. For treated

plants, the corresponding values were 17% and 31%. This increase in the relative abundance of ureides between day 2 and day 6 indicates that the increase in the ureide concentration over the same period, mentioned above, was not attributable to a decrease in xylem sap exudation rate.

Whilst the use of relative ureides overcomes problems associated with variations in exudation rate, it does not inform us, directly, of any effect of nitrate on ureide production or export from the nodules. The major factor contributing to the decline in the relative abundance of ureides in the xylem sap of nitrate treated plants was the appearance of nitrate in the denominator of the equation for calculating relative ureides; (ureides/[ureides + α-amino-N + nitrate]) x 100. Therefore, the correlation between the relative abundance of ureides and the contribution of N_2 fixation to total plant nitrogen may be more a function of an inverse relationship between nitrate uptake and N_2 fixation than a positive relationship between xylem sap ureide content and N_2 fixation.

Xylem sap exudation rate was stimulated by 67% (2 d) and 71% (6 d) in the presence of nitrate (Table 3.2). This may have resulted in dilution of the contents of the exudate and hence an overestimation of the effect of nitrate treatment on N_2 fixation. However, since the magnitude of the effect of nitrate treatment on xylem sap ureide concentration was similar to that obtained using other methods to estimate N_2 fixation, it is unlikely that any such dilution occurred.

Rather it may be that nitrate treatment resulted in a similar increase in both xylem sap exudation rate and the loading of ureides from the nodule into the xylem.

The model proposed by Pate et al. (1969) to describe xylem loading of the products of N_2 assimilation can be summarised as follows: The products of N_2 fixation diffuse into the apoplast of the nodule vascular bundle resulting in a reduction in the water potential and a consequent influx of water which flushes them out of the nodule and into the shoots via the xylem. In other words, water movement into the apoplast follows the establishment of a high concentration of nitrogenous compounds in that compartment. In accordance with this model, a mechanism can be envisaged by which increased uptake of water into the apoplast, driven by the uptake of nitrate ions, could result in increased xylem loading of the products of N_2 fixation. The concentration gradient of ureides between the apoplast of the nodule bundle sheath, the uninfected cells and the bacteroid containing cells, which sustains the flux of these compounds into the apoplast, would be made steeper if water flux through the apoplast was increased. Consequently, increased loading of the products of N_2 fixation into the xylem would occur. Thus it is possible that nitrate treatment increased both xylem sap exudation rate and export of ureides from the nodules.

3.2.3 Nodule Ureide Content

Nodules detached from the root systems used for the acetylene reduction assay (see section 3.2.1) were analysed for ureide content.

The results of this analysis are presented in Table 3.3. There was no significant effect of nitrate on nodule ureide content after 2 d of treatment. After 6 d, however, nitrate treatment resulted in a significant accumulation of ureides in the nodule. In another experiment in which nitrate was supplied at a higher concentration (10 mM) and for a longer period of time (14 d), nitrate-induced accumulation of ureides in the nodule was more marked (Table 3.4). Similar results have been reported previously (Streeter 1985 a, b). It should be noted that the long-term nitrate treatment inhibited nodule growth whereas the short-term treatment did not and the effect of 10 mM nitrate was proportionally greater than that of 5 mM nitrate.

There are several possible explanations for this accumulation of ureides in the nodules. Firstly, ureides may be synthesised from nitrate in either the roots or the nodules or both (Ohyama and Kumazawa 1979). This possibility can probably be discounted, however, because ureide biosynthesis from nitrate has been reported to be very low (Ohyama and Kumazawa 1979). Secondly, ureides may arise from the products of nucleotide breakdown as a result of nitrate-induced nodule senescence. This possibility has not been investigated but ureides have been shown to be synthesised from the products of nucleotide breakdown in the cotyledons of 8 day-old soybean seedlings whereas ureides in the cotyledons of younger plants are derived from purines synthesised de novo (Polayes and Schubert 1984), suggesting that ureide biosynthesis from the products of nucleotide breakdown may be an indicator of senescence. Thirdly, nitrate treatment may inhibit ureide export from the nodules. This possibility appears most likely, especially in view

of the ultrastructural degeneration of nodules which occurs in response to nitrate treatment (Dart and Mercer 1965, Truchet and Dazzo 1982). Ureide biosynthesis from the products of nucleotide breakdown, as a means of salvaging purines, may be important in the longer term whereas inhibition of export of ureides from the nodules is probably more important in the short term.

3.3 Concluding Remarks

The effect of nitrate treatment on N_2 fixation was determined by assaying ARA, $^{15}N_2$ fixation and xylem sap ureide content. The three methods gave similar results, indicating that ARA is a reliable measure of the effect of nitrate treatment on nitrogenase activity of soybean cv. Bragg, whether or not it underestimates N_2 fixation.

Despite the inhibition of N_2 fixation, as a consequence of nitrate treatment, nodule ureide content did not decline. In fact ureides accumulated in the nodules of plants exposed to nitrate for periods of more than about a week. In the short term this may be due to inhibition of ureide export from the nodules by nitrate. In the longer term it could possibly be explained by ureide biosynthesis from the products of nucleotide breakdown in the nodules. The latter would enable recycling of the nitrogen from senescing nodules for use by the rest of the plant.

Table 3.1 The Effect of Nitrate Treatment on Acetylene Reduction Activity and $^{15}N_2$ Fixation. Soybean cv. Bragg plants, inoculated with B. japonicum strain USDA 110, were grown in the absence of combined nitrogen in 8 l pots of vermiculite (10 plants. pot^{-1}) in a naturally-illuminated glasshouse during November/December as described in Chapter 2. Nitrate treatment was commenced 38 DAP with control plants receiving 5 mM KCl and treated 5 mM KNO_3. After 2 d and 6 d of nitrate treatment, three plants from each of two pots were assayed for nitrogenase activity using the comparative acetylene reduction assay (see Chapter 2) and another three plants from the same two pots were assayed for $^{15}N_2$ fixation activity (see Chapter 2). Data are the mean ± SD (n=6). The mass spectrometric analyses for ^{15}N content of the digested plant material (see Chapter 2) were carried out by G.L. Turner, Division of Plant Industry CSIRO, Canberra, ACT, Australia.

	2 d		6 d	
Parameter	control	treated	control	treated
Nitrogenase activity (μmol C_2H_4. h^{-1}. g^{-1} nodule FW)	11.9±3.9	6.6±2.0	13.2±2.6	5.4±2.1
$^{15}N_2$ fixation (μmol N_2. h^{-1}. g^{-1} nodule FW)	3.8±0.8	2.1±0.8	5.8±2.4	2.4±1.7

Table 3.2 The Effect of Nitrate Treatment on the Nitrogen Content of Xylem Sap. Xylem sap was collected (as described in Chapter 2) from the four plants remaining in each pot from the experiment outlined in the legend to Table 3.1. Ureides, nitrate, α-amino-nitrogen and the xylem sap exudation rate were determined as described in Chapter 2. Data are the mean (± SD) of 4-7 replicates.

ND* = Not Detectable

	2 d		6 d	
Parameters	control	treated	control	treated
N-compound concentration (μmol. ml^{-1} xylem sap)				
Ureides	5.6±1.0	1.9±1.0	5.2±1.5	2.8±1.1
Nitrate	ND*	8.0±1.5	ND	5.8±1.4
α-amino-N	1.5±0.5	1.3±0.3	3.0±0.3	0.5±0.7
Xylem sap exudation rate (μl. min^{-1})	7.5±1.8	12.5±3.5	7.8±4.7	13.8±8.8

Table 3.3 The Effect of Nitrate Treatment on Nodule Ureide Content.

Subsamples of nodules detached from the plants assayed for acetylene reduction activity in the experiment outlined in the legend to Table 3.1 were analysed for ureides as described in Chapter 2. Data are the mean ± SD (n=4).

Nitrate treatment	Nodule ureide content (μmol. g^{-1} nodule FW)	
	control	treated
2 d	7.2±1.0	5.9±0.5
6 d	8.5±0.6	12.5±1.2

Table 3.4 The Effect of Long-Term Nitrate Treatment on Nitrogenase Activity, Nodule Fresh Weight and Nodule Ureide Content. The plants were grown as described in the legend to Table 3.1 except that nitrate was supplied at a concentration of 10 mM, the nitrate treatment was continued for 14 d, and the plants were harvested 63 DAP, in mid-February. Nitrogenase activity was determined using the standard acetylene reduction assay (see Chapter 2) and nodule ureide content was assayed as described in Chapter 2. Data are the mean ± SD (n=4).

Parameter	control	treated
Nitrogenase activity (μmol C_2H_4. h^{-1}. g^{-1} nodule FW)	18.7±1.8	0.5±0.2
Nodule fresh weight (g. $plant^{-1}$)	1.76±0.37	0.61±0.22
Nodule ureide content (μmol. g^{-1} nodule FW)	2.62±0.17	10.27±1.13

CHAPTER 4

The Effect of Nitrate Treatment on N_2 and Carbon Metabolism in Soybean cv. Bragg Nodules.

4.1 Introduction

In the short term, treatment of already established soybean - Bradyrhizobium symbioses with nitrate (delayed nitrate treatment) inhibits nitrogenase activity and bacteroid protein synthesis and decreases xylem sap ureide content, without affecting plant or nodule growth (Noel et al. 1978, McClure and Israel 1979, Herridge 1982). However, it is not known what effect this treatment has on the enzymes involved in (1) the assimilation of fixed nitrogen, (2) the provision of carbon skeletons for this process and (3) the provision of respiratory substrates for the bacteroids. This chapter, therefore, examines the effect of delayed nitrate treatment on the activities of some of the enzymes involved in N_2 assimilation and carbon metabolism in soybean nodules. In addition, the effect of delayed nitrate treatment on the spectrum of nodule cytoplasmic proteins is examined using one dimensional SDS-PAGE. The aim of the latter was to correlate any nitrate-induced changes in the SDS-PAGE profile with nitrate-induced changes in enzyme activities.

4.2 Results

4.2.1 Nitrogenase Activity of Nodulated Roots

When expressed on a nodule fresh weight basis, nitrogenase activity of nodulated roots, as determined by acetylene reduction, was inhibited by 48%, 94% and 92% after 2, 7, and 14 d, respectively, of nitrate treatment (Fig. 4.1). Similar, although less marked, reductions were seen in xylem sap ureide concentration (Fig. 4.2). The values were 23% 70% and 88% after 2, 7 and 14 d, respectively.

These results are in some respects different from those presented in Chapter 3 where it was noted that, after 2 d of nitrate treatment, nitrogenase activity was inhibited by 44% (acetylene reduction) and 47% ($^{15}N_2$ fixation) whereas xylem sap ureide concentration declined by 66%. Here nitrogenase activity was inhibited by 48%, which was similar to the results presented earlier. However, xylem sap ureide concentration declined by only 23%. In other words, the decline in ureide concentration was in one case greater and in the other case less than the inhibition of nitrogenase activity. The smaller decline in ureide concentration observed in the more recent experiment may be due to a reduced rate of exudation of xylem sap from the nitrate treated plants resulting in concentration of the contents of the sap of these plants.

There was no significant effect of nitrate treatment on either the fresh weight (Table 4.1) or total soluble protein content (Fig. 4.3) of nodules for up to at least 7 d of nitrate treatment. Leghaemoglobin

content of the nodules was similarly not affected for up to at least 7 d of nitrate treatment (Fig. 4.4). By 14 d, however, nodule fresh weight and leghaemoglobin content had declined markedly in response to nitrate treatment.

4.2.2 Nitrogen Content of the Xylem Sap.

The effect of nitrate treatment on the nitrogen content of the xylem sap is illustrated in Table 4.2. The nitrogen compounds assayed were nitrate, α-amino-N and the ureides, allantoin and allantoic acid. The sum of these compounds accounts for 99% of the nitrogen in the xylem sap of soybean (McClure and Israel 1979). Ureides were the most abundant nitrogen compounds in the xylem sap of control (N_2 dependent) plants, constituting 60%-90% of the nitrogen present, whereas nitrate was the most abundant nitrogen compound in the xylem sap of nitrate treated plants, constituting 50%-60% of the nitrogen present. The reduction in the ureide content of the xylem sap in response to nitrate treatment, along with the appearance of nitrate and an increase in the α-amino-N concentration, resulted in ureides constituting only 19% (after 2 d) and 10% (after 7 d) of the nitrogen in the xylem sap of nitrate treated plants. The decline in the relative abundance of ureides (ureides expressed as proportion of total nitrogen) in the xylem sap, following nitrate treatment, was consistent with the inhibition of nitrogenase activity (Fig. 4.1).

There was an increase in α-amino-N concentration in the xylem sap in response to nitrate treatment indicating some nitrate reduction in

the roots (Table 4.2). The concentration of α-amino-N compounds in the xylem sap of nitrate treated plants in the experiment shown in Table 4.2 was higher than it was in the experiment displayed in Table 3.2. This can probably be explained by the higher nitrate concentration employed in the experiment shown in Table 4.2. In other words, increasing the nitrate concentration supplied to soybean plants may favour increased nitrate reduction in the roots.

The similarity between Table 3.2 and Table 4.2 in the concentration of nitrate in the xylem sap, despite the difference in the concentration of nitrate supplied to the plants may possibly be explained by increased conversion of nitrate to α-amino-N compounds in the experiment shown in Table 4.2.

The increase in ureide concentration in the xylem sap of nitrate treated plants between day 2 and day 6, shown in Table 3.2 is in apparent conflict with the decrease between day 2 and day 7 shown in Table 4.2. This conflict is probably due to differences between plants in xylem sap exudation rate.

4.2.3. Enzymes of Ammonia Assimilation and Ureide Biosynthesis.

Despite the marked inhibition of nitrogenase activity (Fig. 4.1) and the reduction in xylem sap ureide content (Fig. 4.2), there was no significant effect of nitrate treatment on <u>in vitro</u> activity of glutamine synthetase (Table 4.3). Xanthine dehydrogenase (XDH) (Fig. 4.5), uricase (Fig. 4.6) and allantoinase (Fig. 4.7) activities were

similarly not affected for up to at least 7 d. In contrast, glutamine oxoglutarate aminotransferase (GOGAT) activity was inhibited by 54% after 7 d although it was not significantly affected after only 2 d of nitrate treatment (Fig. 4.8). XDH activity was somewhat inhibited after 14 d of nitrate treatment whilst the other enzymes were not affected. GS and GOGAT catalyse the initial reactions in the assimilation of the ammonia exported by the bacteroids (see Boland et al. 1980). XDH, uricase and allantoinase catalyse the oxidative degradation of purines to yield ureides (see Schubert and Boland 1984).

4.2.4 Nitrogenase Activity of Isolated Bacteroids.

The results of a typical experiment in which nitrogenase (acetylene reduction) activity of bacteroids isolated from the nodules of nitrate treated plants was compared with that of bacteroids isolated from the nodules of control plants are presented in Table 4.4. Activity was assayed in the presence of 0.2%, 0.5%, 1.0%, 1.5% or 2% (v/v) O_2 in N_2 or argon and either endogenous substrates or endogenous substrates supplemented with malate. Only the results obtained at the optimal O_2 concentration are shown and discussed. At this O_2 concentration, appreciable activity was observed with endogenous substrates alone. However, when malate was added there was an almost four-fold stimulation. Activity of isolated bacteroids, in the presence of added malate, was the same as that of nodulated roots when both activities were expressed on a bacteroid protein basis. This indicates that little activity was lost during bacteroid isolation. The results also suggest that oxidation of endogenous substrates is not sufficient to support

nitrogenase activity *in vivo*. That is, in intact nodules, bacteroids must be being continually supplied with substrates from the host cytoplasm.

After 2 d of nitrate treatment, nitrogenase activity of nodulated roots was inhibited by 70%. In contrast, nitrogenase activity of bacteroids isolated from nitrate-inhibited nodules was identical to that of bacteroids isolated from control nodules. However, after 7 d of nitrate treatment, nitrogenase activity of isolated bacteroids was also severely inhibited; activity dependent on endogenous substrates was completely abolished and malate dependent activity was inhibited by 79%. This was coincident with a 90% inhibition of nitrogenase activity of nodulated roots.

4.2.5 Enzymes of Carbon Metabolism in the Nodules

After 7 d of nitrate treatment, nitrogenase activity was inhibited by 89% whereas alkaline invertase activity was reduced by only 19% and fructokinase activity by only 25% (Table 4.5). The reduction in fructokinase activity was barely statistically significant (T-test, $p=0.05$) and the reduction in alkaline invertase activity was not significant. The effect of nitrate treatment on PEP carboxylase activity was more marked, however, but the inhibition was only 43% as compared with 81% for nitrogenase activity (Table 4.6). Alkaline invertase and fructokinase catalyse the initial steps in sucrose degradation in soybean nodules (Morell and Copeland 1984, 1985). PEP

carboxylase is involved in the synthesis of carbon skeletons for ammonia assimilation and substrates for bacteroid respiration (see Gadal 1983).

4.2.6 SDS-PAGE Analysis of Nodule Cytoplasmic Proteins

Bacteroid-free nodule extracts were analysed by one dimensional SDS-PAGE (Fig. 4.9). Nodules were harvested from plants which had been treated with nitrate for up to 14 d. Although nitrogenase activity of nodulated roots was markedly inhibited, there were no differences between the SDS-PAGE profiles of nodules taken from control and treated plants.

4.3 Discussion

Despite the marked decline in nitrogenase activity and the ureide content of xylem sap, there was no effect of short term (2 d), delayed nitrate treatment on (1) nitrogenase activity of isolated bacteroids, (2) in vitro activity of enzymes involved in ammonia assimilation, ureide biosynthesis and carbon metabolism in the nodules or (3) leghaemoglobin and total soluble protein content of the nodules. After 7 d of nitrate treatment, however, nitrogenase activity of isolated bacteroids and extractable GOGAT activity were markedly inhibited. The other enzymes and the soluble protein content of the nodules remained unaffected for at least 7 d. These results are consistent with the previously reported observation that the inhibitory effect of delayed nitrate treatment on nitrogenase activity was reversible for up to at least 10 d after the commencement of treatment (Gibson 1976).

Furthermore, they indicate that the nitrate-induced decline in nitrogenase activity does not involve the loss of the enzymic capacity of nodules to assimilate N_2 and metabolise carbon. Nor does it involve the loss of leghaemoglobin. However, the possibility of nitrite binding to and reversibly inactivating nitrogenase and/or leghaemoglobin (Rigaud and Puppo 1977, Trinchant and Rigaud 1980) cannot be ruled out.

The observation that isolated bacteroids retained full N_2 fixation capacity, whilst nitrogenase activity of nodulated roots was markedly inhibited, confirms previous results obtained with pea and soybean (Houwaard 1980, McNeil et al. 1984). However, it is in conflict with results obtained with French bean (Trinchant and Rigaud 1984). This conflict may arise because soybean and French bean differ from one another in their susceptibility to nitrate inhibition. Alternatively, the mechanism of nitrate inhibition may differ between soybean and French bean. A recent direct comparison between these species showed, firstly, that French bean is indeed more susceptible to nitrate inhibition (Wasfi and Prioul 1986). Secondly, in French bean nodules, $^{14}CO_2$ fixation declined coincidentally with the nitrate induced decline in nitrogenase activity, whereas, in soybean nodules the decline in $^{14}CO_2$ fixation was delayed relative to the decline in nitrogenase activity. The authors concluded that nitrite may mediate the inhibitory effect of nitrate in soybean whereas a decline in photosynthate allocation to the nodules may be involved in French bean. This difference between soybean and French bean suggests that nitrite levels and compartmentation in the nodules may differ between the two species.

Nitrate treatment has been shown to inhibit protein synthesis in both the bacteroid and the plant fraction of pea and soybean nodules (Bisseling et al. 1978, Noel et al. 1982). Therefore, the absence of any effect of nitrate treatment on total soluble protein content of the nodules (this study), indicates that nodule protein turnover is slow. Since the loss of nitrogenase activity of isolated bacteroids preceded the loss of in vitro activity of the plant enzymes assayed, this suggests that the half-life of bacteroid proteins may be shorter than that of plant derived nodule proteins. This is borne out by the observations of Coventry and Dilworth (1976) who determined a half-life for bacteroid proteins of 5 d and for plant derived nodule proteins of 15-20 d.

The enzymes assayed in this study are amongst the most abundant proteins in soybean nodules (see Verma et al. 1983). Consequently, they should be detectable using SDS-PAGE. Therefore, the lack of any effect of nitrate treatment on the in vitro activities of these enzymes is consistent with the absence of any effect on the SDS-PAGE spectrum of nodule cytoplasmic proteins.

The inhibitory effect of nitrate treatment of nodule protein synthesis (see above) may occur either at the level of transcription or at the translational level. Therefore, the spectrum of in vitro translation products of nodule mRNA may be altered in response to nitrate treatment. Given the apparently long half-lives of nodule proteins (see above), nitrate induced changes in the in vitro translation products may be seen before changes in total soluble nodule

proteins. Consequently an investigation of nitrate-induced changes in the levels of *in vitro* translation products of nodule mRNA may prove more fruitful in terms of an analysis of nitrate inhibition. This remains to be seen.

The absence of any effect of delayed nitrate treatment on the enzymes involved in ureide biosynthesis (this study) was consistent with the results obtained when nitrate was supplied from the time of planting (Zengbe *et al*. 1984). In contrast, when well nodulated cowpea plants were transferred to an N_2-free atmosphere (80% Ar:20% 0_2), the activities of these enzymes declined markedly (Atkins *et al*. 1984 a). The major difference between the nitrate treatment and the Ar:0_2 treatment is that the former did not completely inhibit N_2 fixation whereas the latter did. These results indicate that ammonia production by the bacteroids is required to maintain the enzymes involved in ureide biosynthesis in the nodules.

The inhibitory effect of nitrate treatment on alkaline invertase and fructokinase activity, enzymes involved in sucrose breakdown in soybean nodules (Morrell and Copeland 1984), was small relative to the large effect on nitrogenase activity of nodulated roots. The reduction in PEP carboxylase activity, following nitrate treatment, was greater than the reduction in either alkaline invertase or fructokinase activity, but still less than the reduction in nitrogenase activity. Therefore, it is unlikely that either sucrose degradation or CO_2 fixation in the nodules was limiting nitrogenase activity of nitrate treated plants. Similarly, a less than 10% decline in total nodule

carbohydrate content, associated with an 80% inhibition of nitrogenase activity, has been reported for soybean (Streeter 1981). These results are consistent with the minor importance of a decline in photosynthate translocation to the nodules, in relation to the nitrate-induced decline in nitrogenase activity in soybean (Wasfi and Prioul 1986).

In conclusion, although this study failed to identify the primary cause of the nitrate-induced decline in nitrogenase activity it did show that neither irreversible inactivation of nitrogenase nor a reduction in the activity of the major enzymes involved in ammonia assimilation and sucrose degradation in soybean nodules is involved. The eventual loss of these enzymes is probably a result of, rather than being the cause of, nitrate inhibition of N_2 fixation.

Fig. 4.1 The Effect of Nitrate Treatment on Nitrogenase Activity.

Soybean cv. Bragg plants, inoculated with B. japonicum strain CB1809, were grown in the absence of combined nitrogen in 8 l pots of vermiculite (10 plants. pot^{-1}), in a naturally illuminated glasshouse during December/January as described in Chapter 2. Nitrate treatment was commenced 41 DAP with control plants receiving 10 mM KCl and treated 10 mM KNO_3 for up to 14 d. The plants to be assayed for nitrogenase activity were all taken from a single pot and xylem sap was collected from plants in another equivalent pot. The standard acetylene reduction assay was used to determine nitrogenase activity (see Chapter 2). Ureides in the xylem sap were analysed as described in Chapter 2. Each point is the mean of five replicates, each consisting of two plants. The vertical bars represent the SD of the mean.

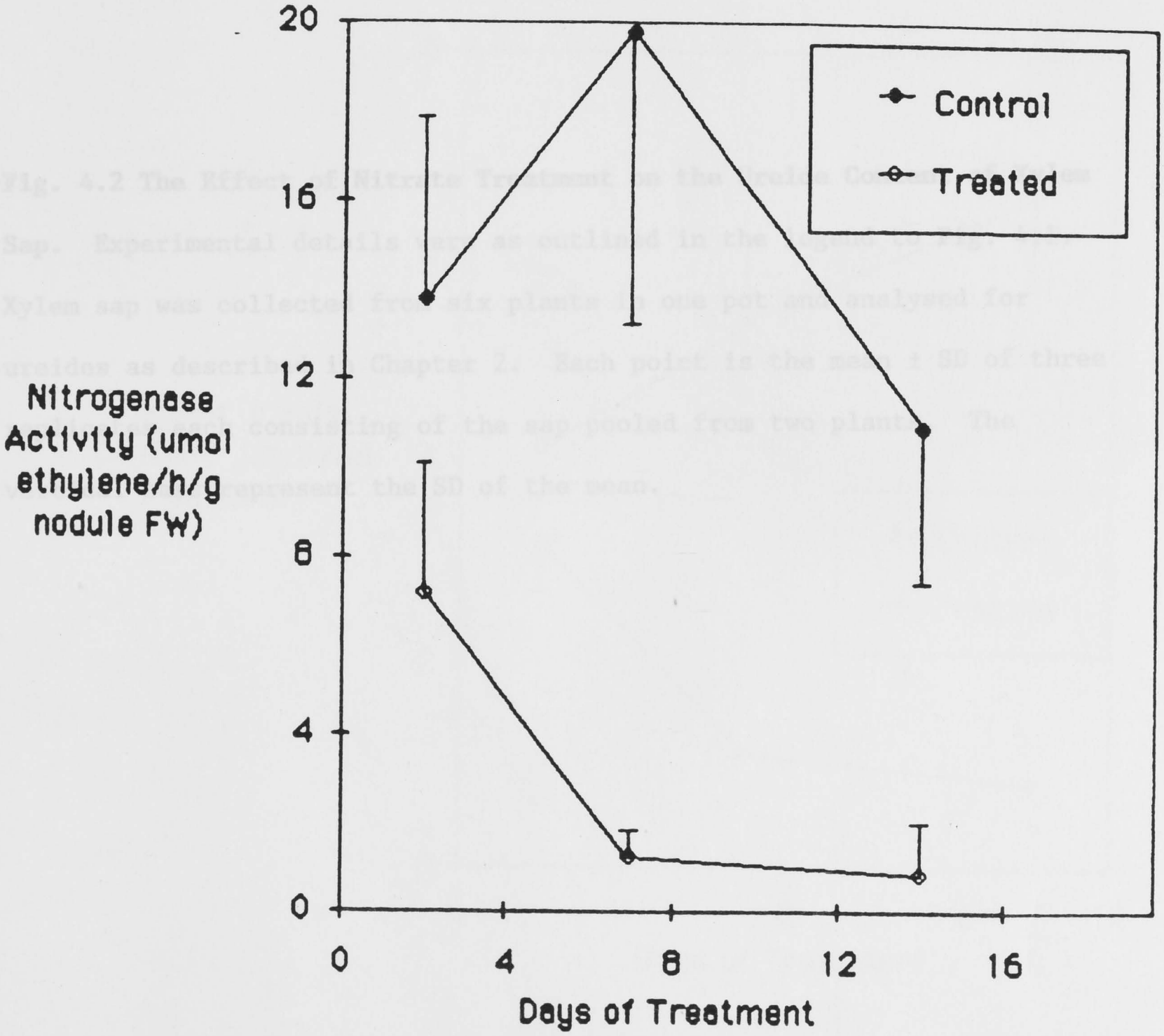

Nitrogenase Activity (umol ethylene/h/g nodule FW)
20
16
12
8
4
0
0
4
8
12
16
Days of Treatment
Control
Treated

Fig. 4.2 The Effect of Nitrate Treatment on the Ureide Content of Xylem Sap. Experimental details were as outlined in the legend to Fig. 4.1. Xylem sap was collected from six plants in one pot and analysed for ureides as described in Chapter 2. Each point is the mean ± SD of three replicates each consisting of the sap pooled from two plants. The vertical bars represent the SD of the mean.

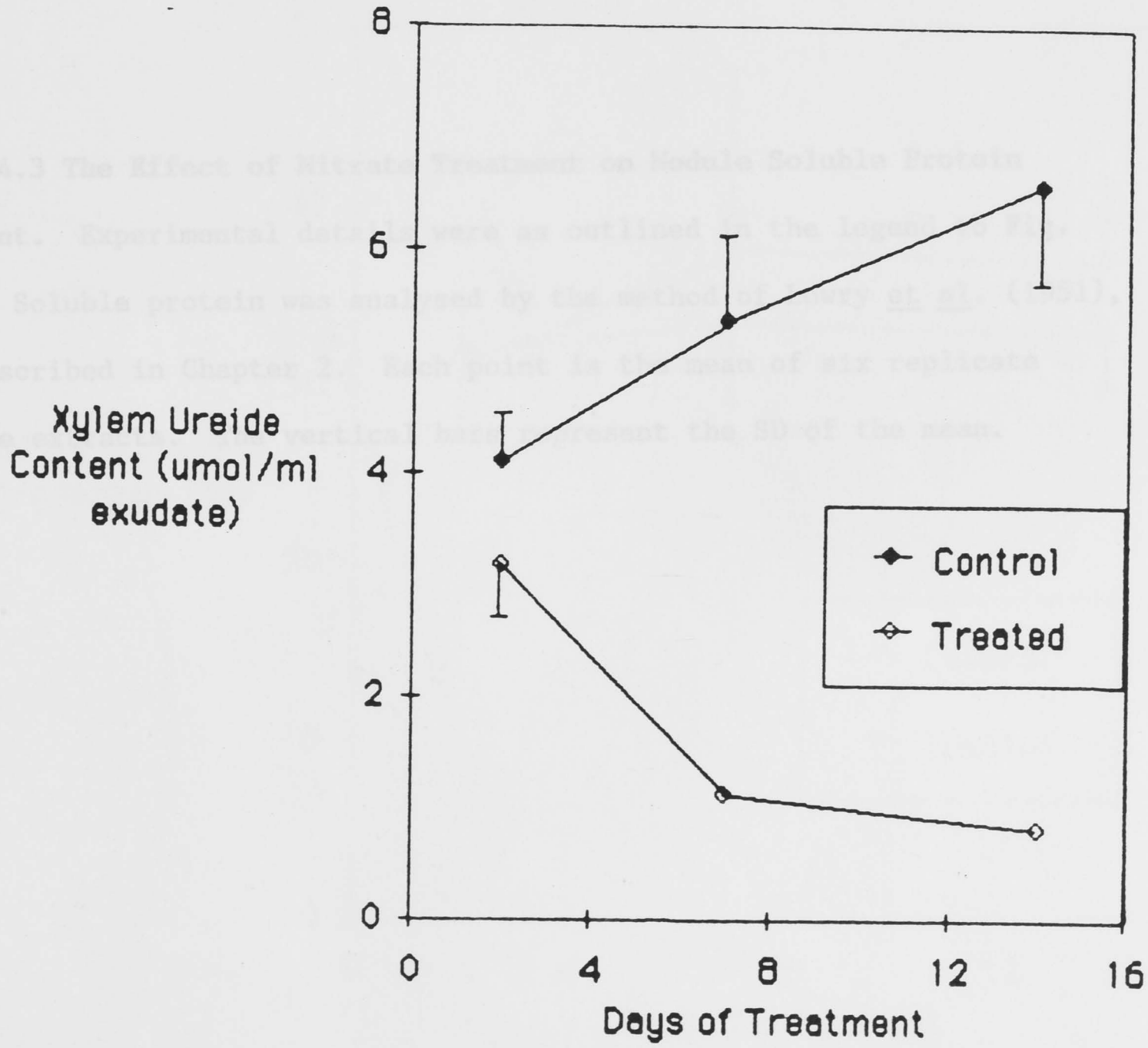

Xylem Ureide Content (umol/ml exudate)
8
6
4
2
0
0
4
8
12
16
Days of Treatment
Control
Treated

Fig. 4.3 The Effect of Nitrate Treatment on Nodule Soluble Protein Content. Experimental details were as outlined in the legend to Fig. 4.1. Soluble protein was analysed by the method of Lowry et al. (1951), as described in Chapter 2. Each point is the mean of six replicate nodule extracts. The vertical bars represent the SD of the mean.

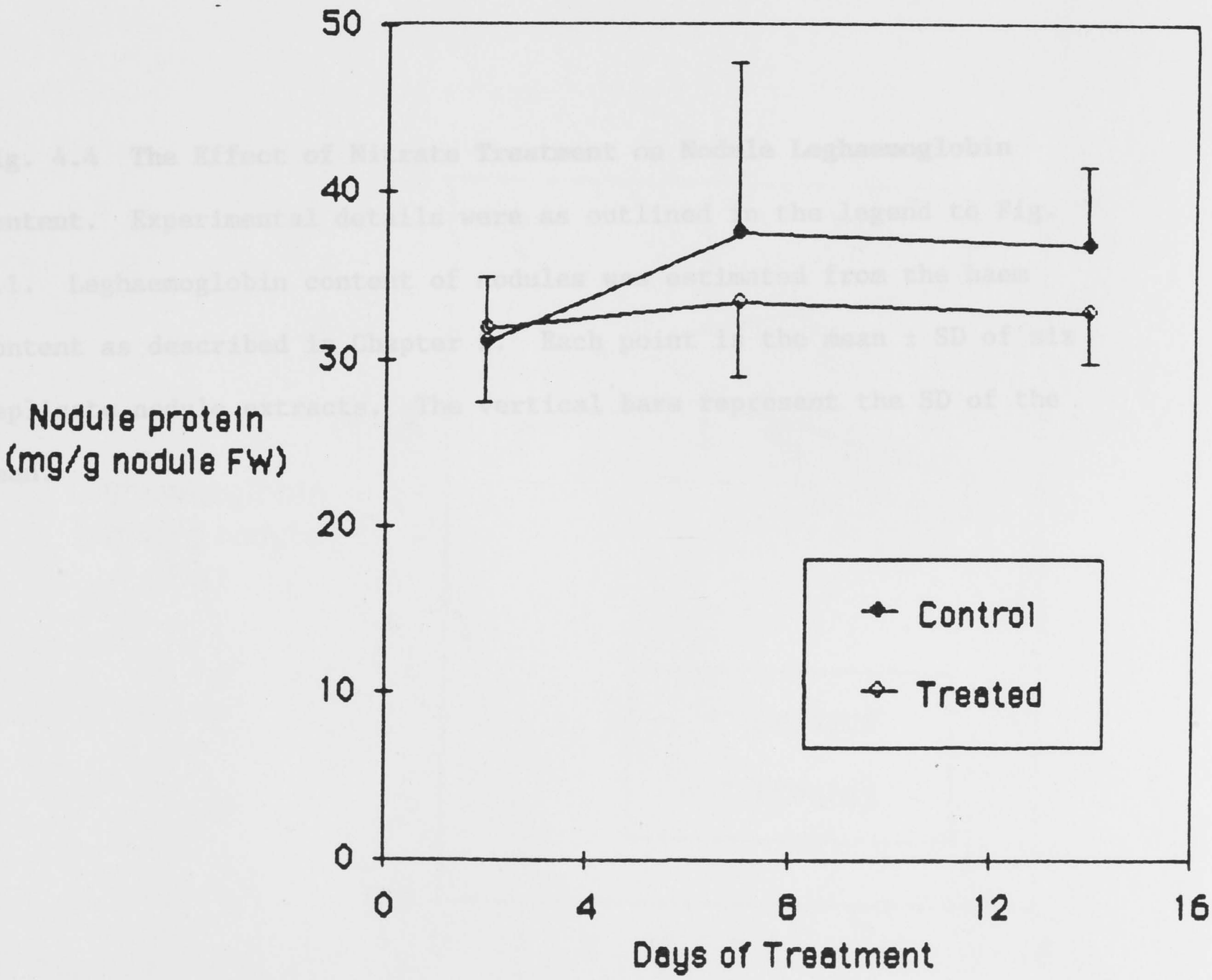

50
40
30
20
10
0
Nodule protein
(mg/g nodule FW)
Control
Treated
0
4
8
12
16
Days of Treatment

Fig. 4.4 The Effect of Nitrate Treatment on Nodule Leghaemoglobin Content. Experimental details were as outlined in the legend to Fig. 4.1. Leghaemoglobin content of nodules was estimated from the haem content as described in Chapter 2. Each point is the mean ± SD of six replicate nodule extracts. The vertical bars represent the SD of the mean.

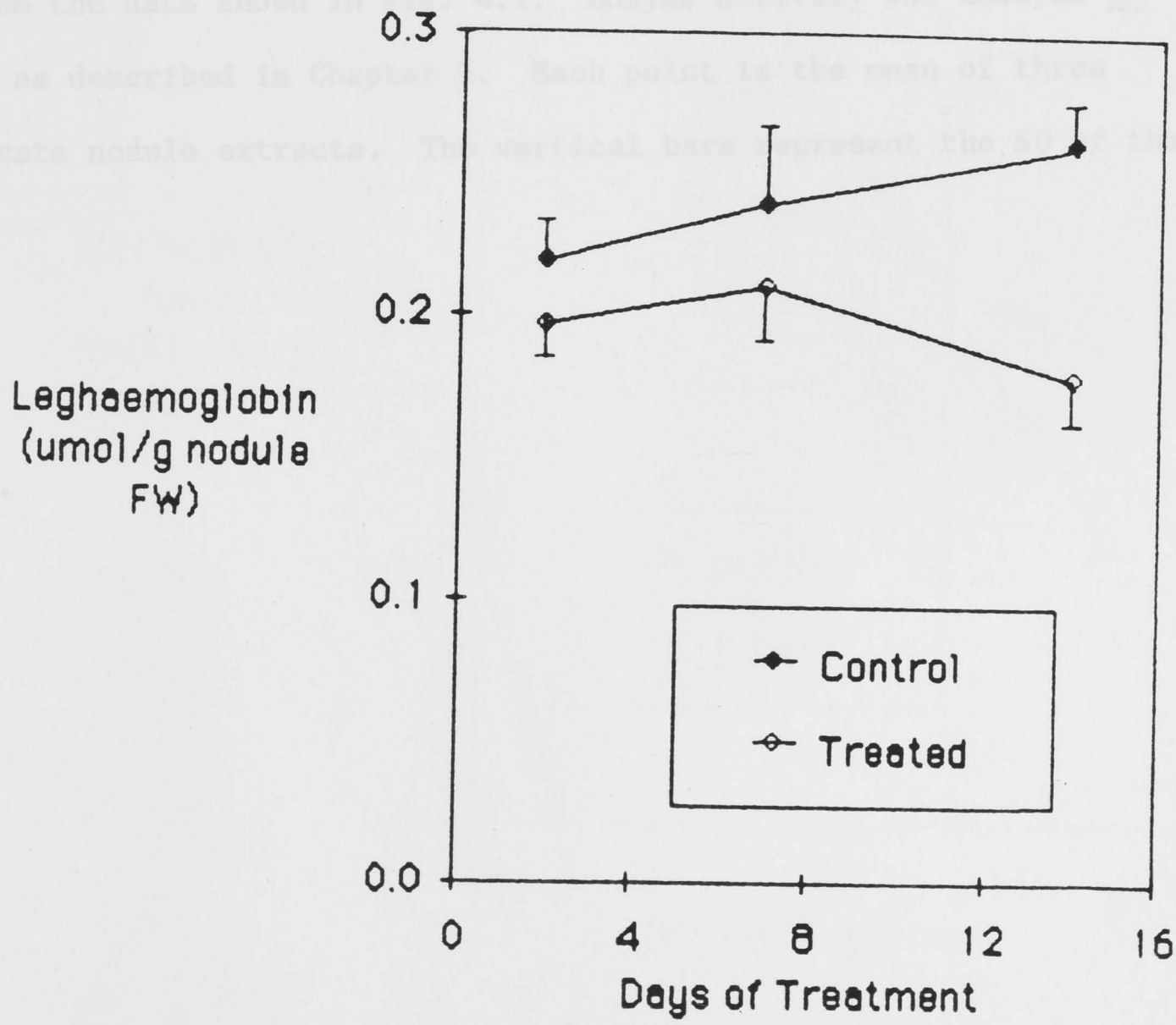

0.3
0.2
0.1
0.0
Leghaemoglobin (umol/g nodule FW)
Control
Treated
0
4
8
12
16
Days of Treatment

Fig. 4.5 The Effect of Nitrate Treatment on Nodule Xanthine Dehydrogenase Activity. Nodules were harvested from the plants used to produce the data shown in Fig. 4.1. Enzyme activity was assayed in vitro as described in Chapter 2. Each point is the mean of three replicate nodule extracts. The vertical bars represent the SD of the mean.

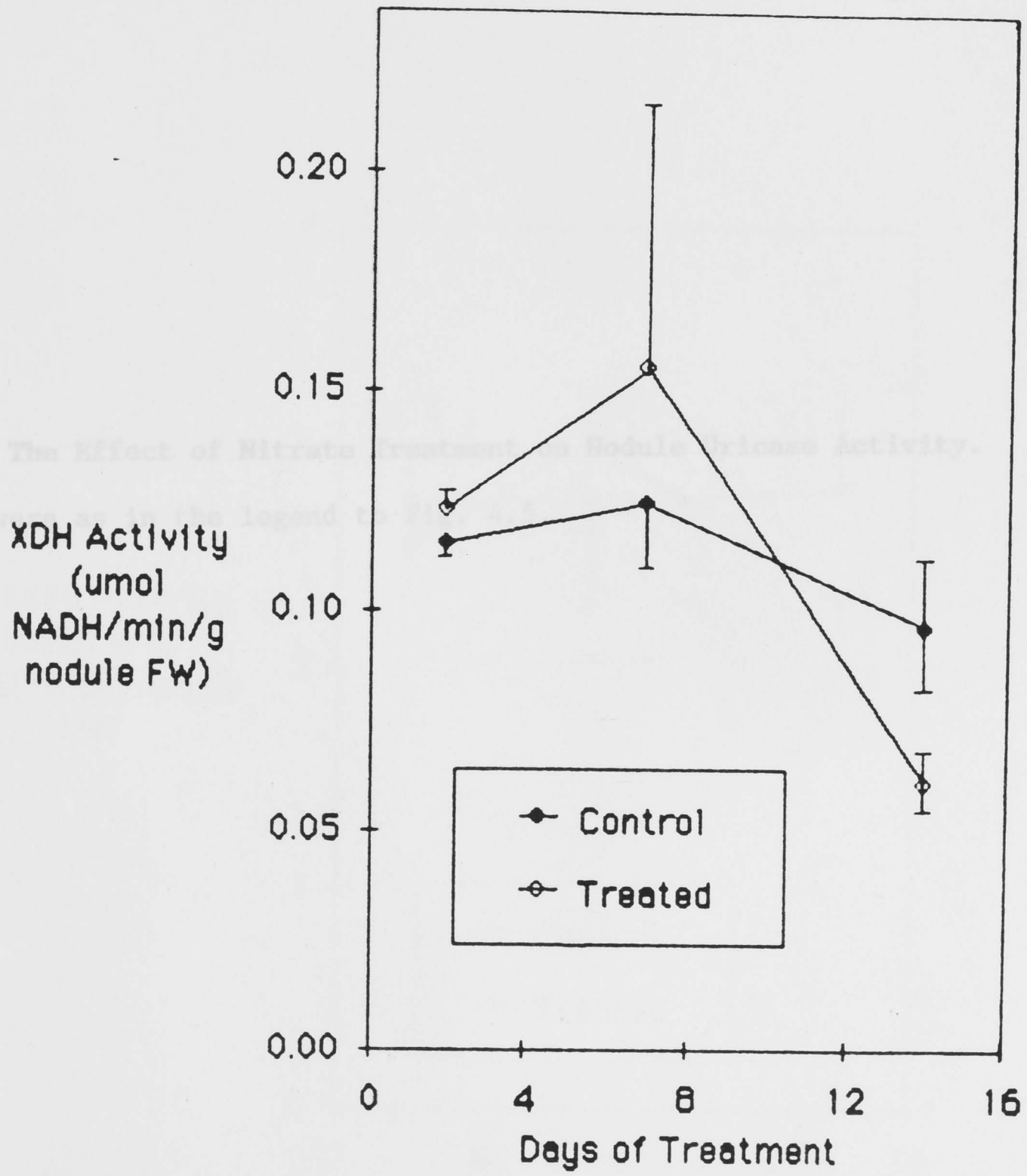

XDH Activity
(umol
NADH/min/g
nodule FW)
0.20
0.15
0.10
0.05
0.00
0
4
8
12
16
Days of Treatment
Control
Treated

Fig. 4.6 The Effect of Nitrate Treatment on Nodule Uricase Activity.

Details were as in the legend to Fig. 4.5.

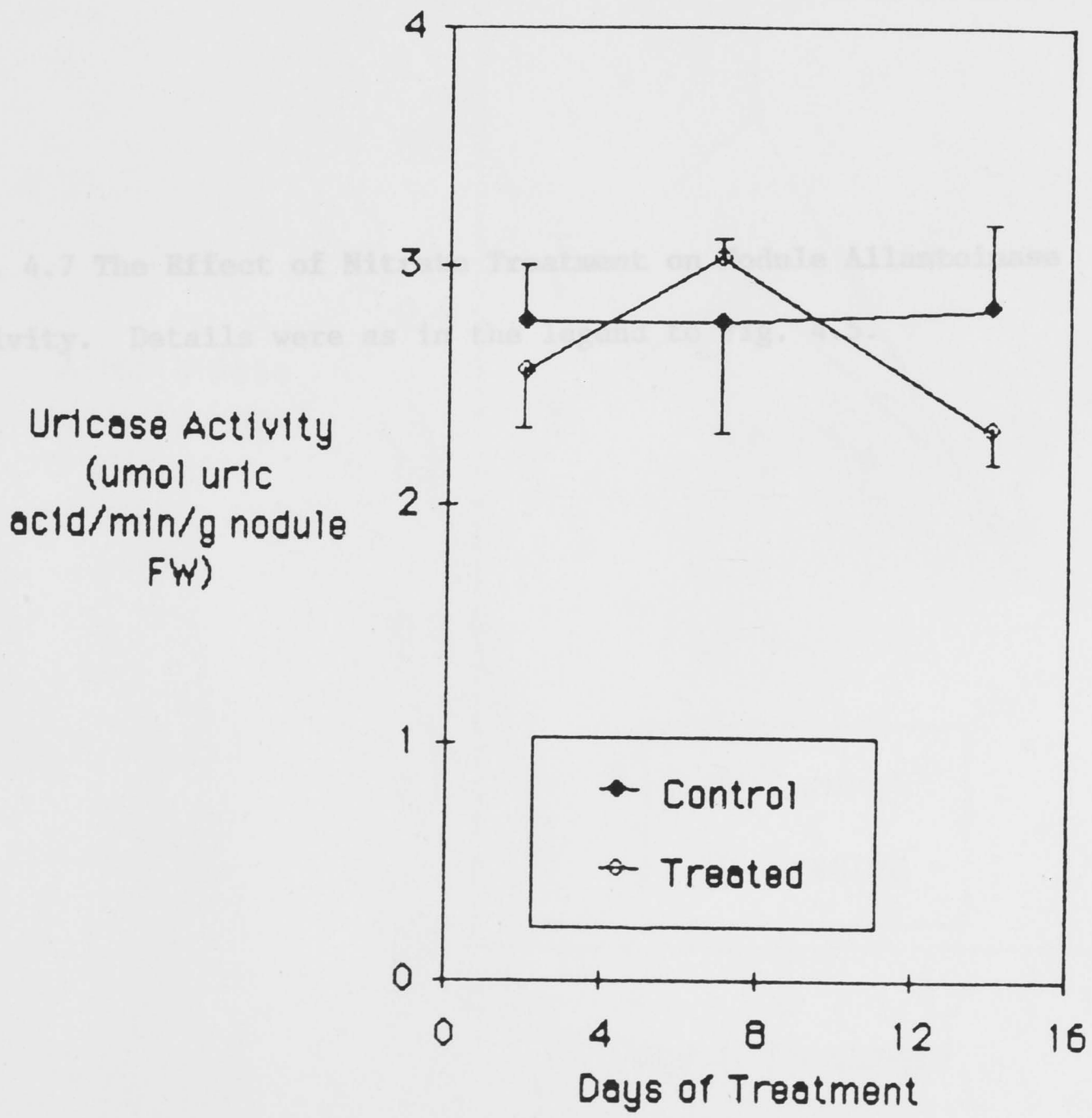

Uricase Activity
(umol uric
acid/min/g nodule
FW)
4
3
2
1
0
Control
Treated
0
4
8
12
16
Days of Treatment

Fig. 4.7 The Effect of Nitrate Treatment on Nodule Allantoinase Activity. Details were as in the legend to Fig. 4.5.

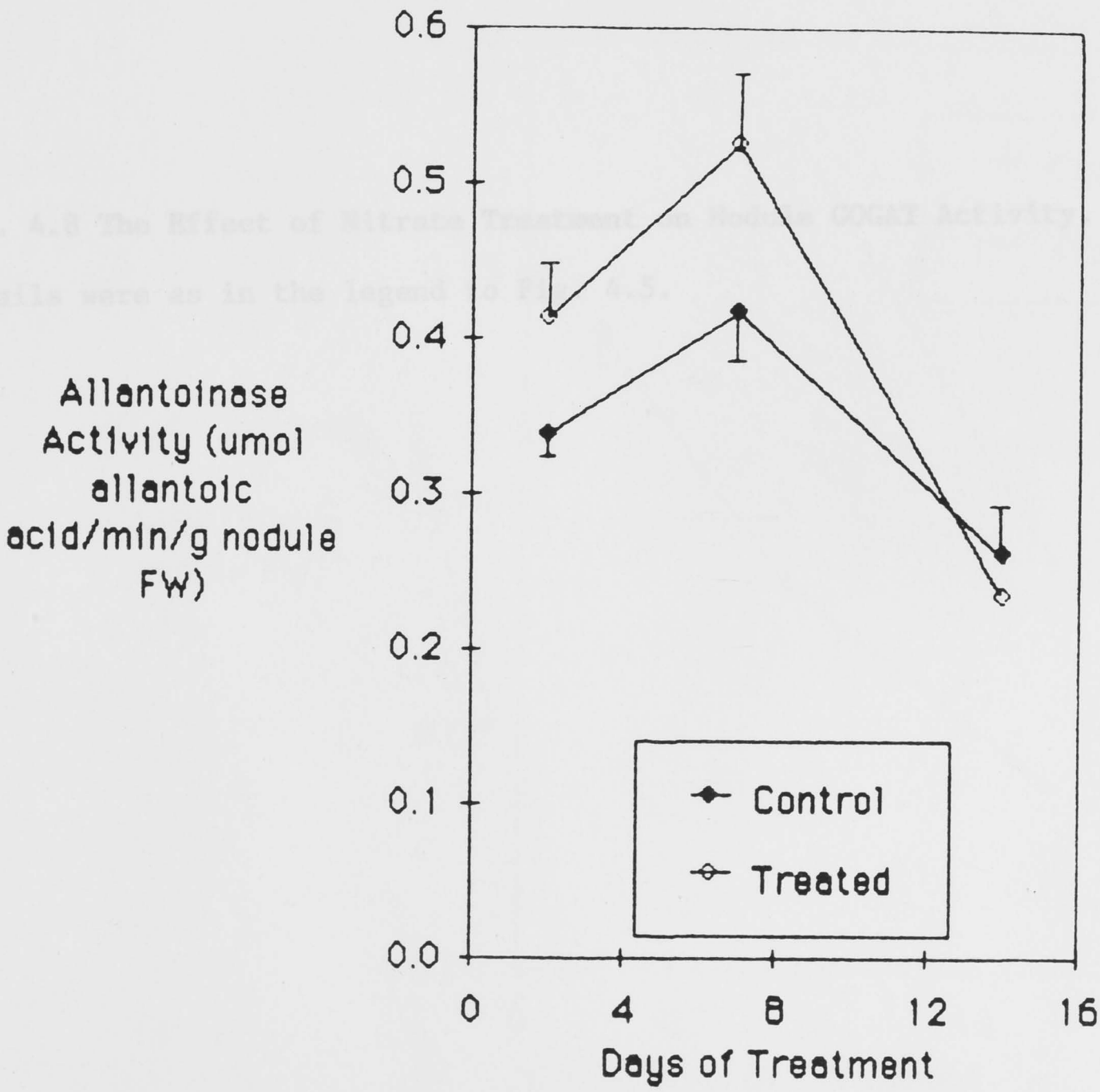

Allantoinase Activity (umol allantoic acid/min/g nodule FW)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
4
8
12
16
Days of Treatment
Control
Treated

Fig. 4.8 The Effect of Nitrate Treatment on Nodule GOGAT Activity.

Details were as in the legend to Fig. 4.5.

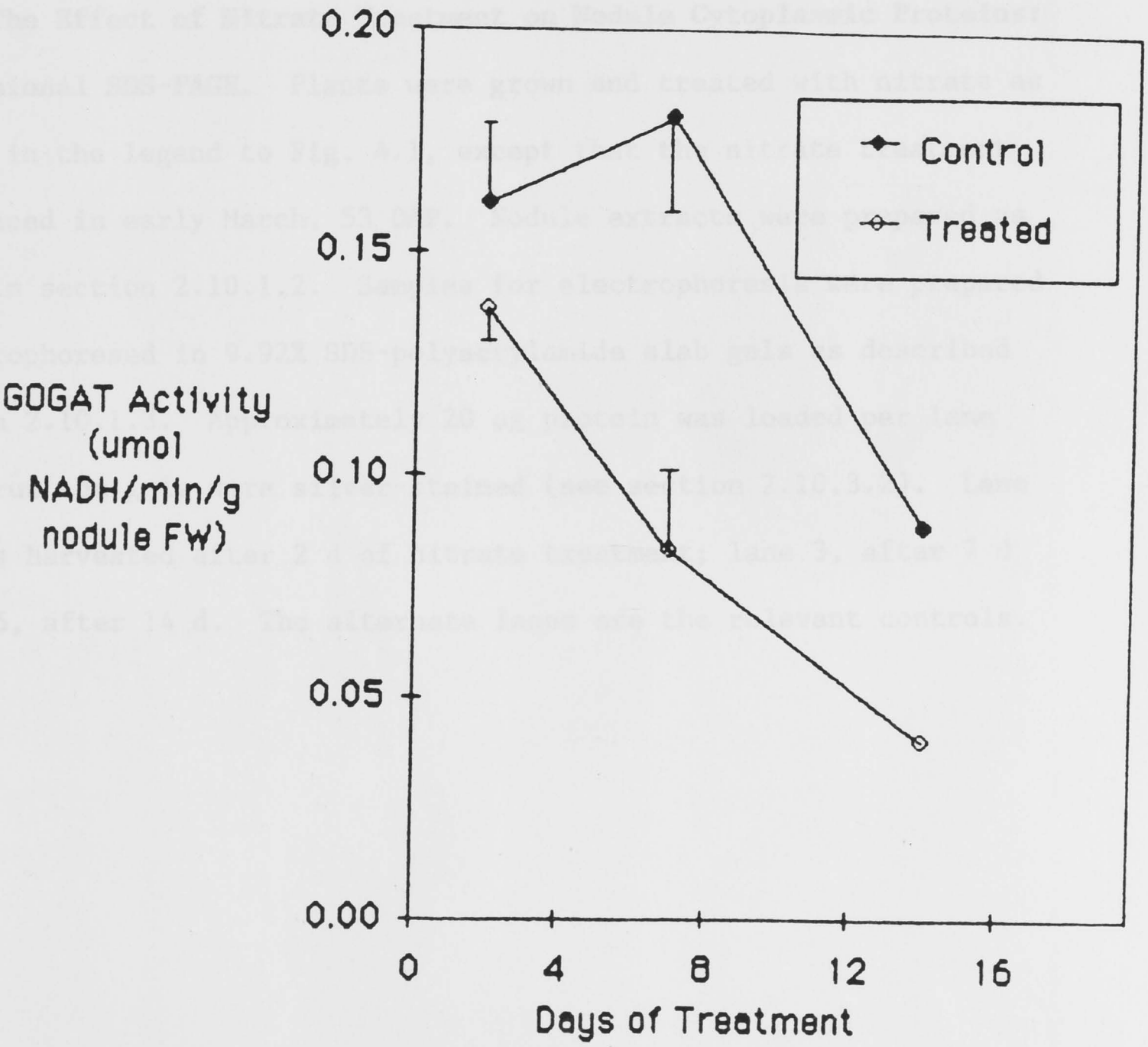

0.20
0.15
0.10
0.05
0.00
GOGAT Activity
(umol
NADH/min/g
nodule FW)
Control
Treated
0
4
8
12
16
Days of Treatment

Fig. 4.9 The Effect of Nitrate Treatment on Nodule Cytoplasmic Proteins: One Dimensional SDS-PAGE. Plants were grown and treated with nitrate as described in the legend to Fig. 4.1, except that the nitrate treatment was commenced in early March, 53 DAP. Nodule extracts were prepared as outlined in section 2.10.1.2. Samples for electrophoresis were prepared and electrophoresed in 9.92% SDS-polyacrylamide slab gels as described in section 2.10.1.3. Approximately 20 µg protein was loaded per lane and once run the gels were silver-stained (see section 2.10.3.2). Lane 1, nodules harvested after 2 d of nitrate treatment; lane 3, after 7 d and lane 5, after 14 d. The alternate lanes are the relevant controls.

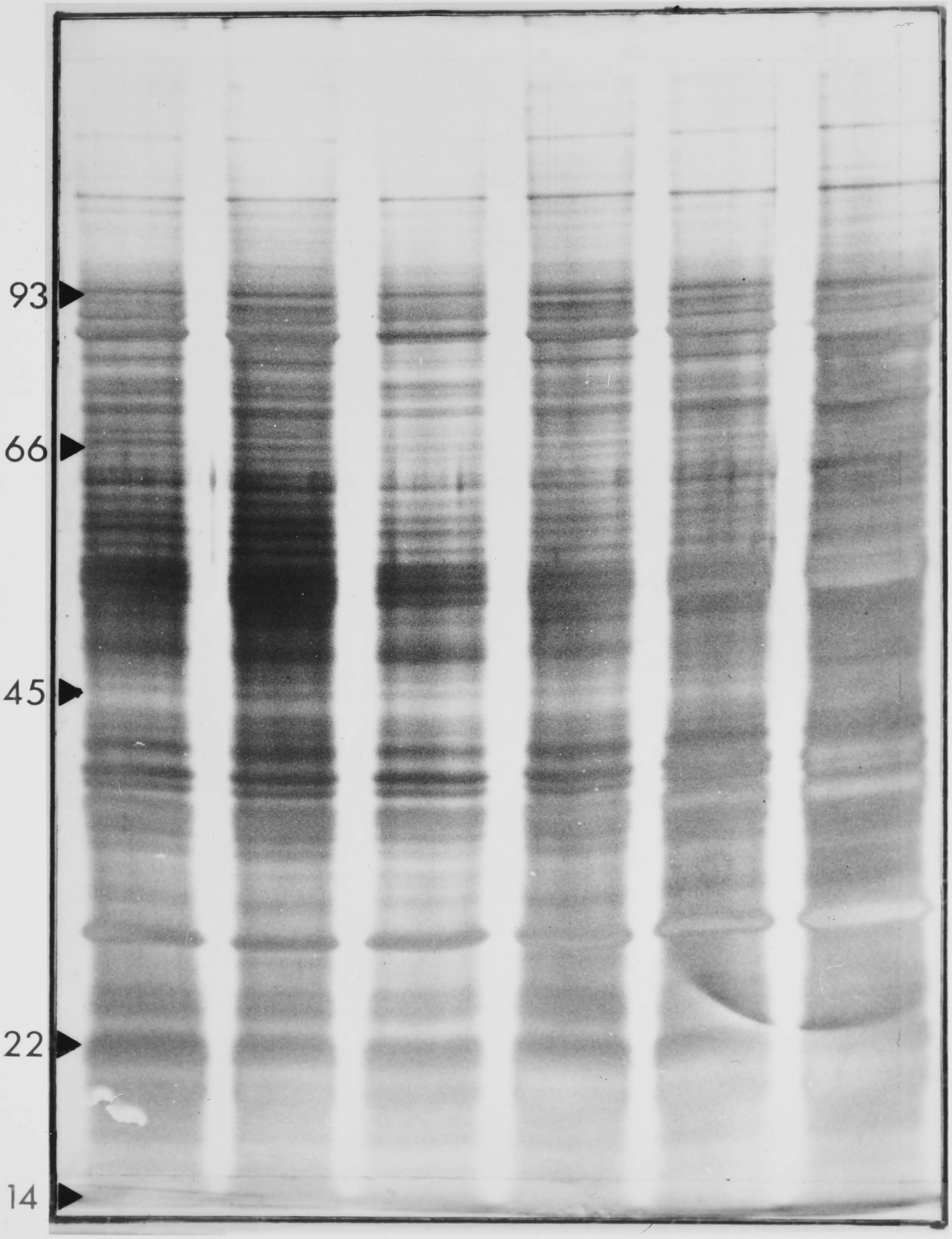

93
66
45
22
14
MW x 10^{-3}

Table 4.1 The Effect of Nitrate Treatment on Nodule Fresh Weight.

Experimental details were as outlined in the legend to Figure 4.1. Nodules were detached from the plants assayed for nitrogenase activity and weighed immediately. Data are the mean ± SD of five replicates, each consisting of two plants.

Days of treatment	Nodule Fresh Weight (g. plant^{-1})	
	control	treated
2	0.27 ± 0.08	0.28 ± 0.08
7	0.57 ± 0.14	0.35 ± 0.09
14	0.33 ± 0.07	0.13 ± 0.03

Table 4.2 The Effect of Nitrate Treatment on Xylem Sap Nitrogen Content.

Experimental details were as outlined in the legend to Figure 4.1. Nitrate, ureides and α-amino-N were analysed as described in Chapter 2. Data are the mean ± SD of three replicates each consisting of the sap pooled from two plants.

*ND = Not Detectable

Parameter		2 d	7 d	14 d
Nitrate (μmol. ml^{-1} xylem sap)				
	control	*ND	ND	ND
	treated	7.9±0.2	4.8±0.6	4.5±0.2
Ureides (μmol. ml^{-1} xylem sap)				
	control	4.1±0.4	5.4±0.8	6.6±0.9
	treated	3.2±0.5	1.1±0.1	0.8±0.1
α-Amino-N (μmol. ml^{-1} xylem sap)				
	control	2.1±0.5	0.8±0.2	2.1±0.3
	treated	5.4±0.3	5.6±0.9	6.4±0.4

Table 4.3 The Effect of Nitrate Treatment on Nodule Glutamine Synthetase Activity. Nodules were harvested from the plants used to produce the data shown in Fig. 4.1. Glutamine synthetase activity was assayed *in vitro* as described in Chapter 2. Data are the mean ± SD of three replicate nodule extracts.

Days of Treatment	Glutamine synthetase activity (μmol glutamyl hydroxamate. $min^{-1} \cdot g^{-1}$ nodule fresh weight)	
	control	treated
7	14.49 ± 0.13	13.88 ± 0.18
14	11.73 ± 0.22	9.13 ± 0.12

Table 4.4 The Effect of Nitrate Treatment on Nitrogenase Activity of Nodulated Roots and Isolated Bacteroids. Experimental details were as outlined in the legend to Fig. 4.1 except that the plants were grown in sand during January/February and were harvested 8-9 weeks after planting. Bacteroid protein content of the nodules was determined using β-HBDH as a bacteroid specific marker (see Chapter 2) and bacteroids were isolated from the nodules and assayed for nitrogenase activity as described in Chapter 2. The activity of β-HBDH was 80 nmol NAD. min^{-1}. mg^{-1} protein and did not change during the period of treatment, nor upon nitrate application. Bacteroid protein content of the nodules was 34.2 ± 2.3 mg bacteroid protein. g^{-1} nodule fresh weight (± SD, n=4) and was unaffected by nitrate treatment. The bacteroid nitrogenase data shown, were obtained at the optimal O_2 concentration for activity which was determined separately for each preparation (see Materials and Methods). Data are the mean ± SD (n=5).

	2 d		7 d	
Nitrogenase activity	control	treated	control	treated
Nodulated roots				
μmol C_2H_4. h^{-1}. g^{-1} nodule FW	11.3±3.4	3.4±1.5	16.2±0.5	1.5±0.9
nmol C_2H_4. h^{-1} · mg^{-1} bacteroid protein	336±101	106±46	432±13	46±27
Isolated bacteroid (nmol C_2H_4. h^{-1}. mg^{-1} bacteroid protein)				
Endogenous	127±15	135±54	51±6	0
5 mM Malate	479±68	439±27	453±14	96±20

Table 4.5 The Effect of Nitrate Treatment on Invertase and Fructokinase Activity. Plants were grown as described in the legend to Fig. 4.1 except that the nitrate treatment period was 7 d and nodules were harvested in December, 42 DAP. Four nodulated root systems from each of two pots were assayed for nitrogenase activity using the standard acetylene reduction assay (see Chapter 2). Data are the mean ± SD (n=4). Nodules detached from the remaining plants were assayed for invertase and fructokinase activity as described in Chapter 2. The same nodule extracts were used for both enzymes. Data are the mean ± SD of four replicate nodule extracts.

Enzyme activity	control	treated
Nitrogenase (μmol C_2H_4. h^{-1}. g^{-1} nodule FW)	15.5±1.8	1.7±0.2
Invertase (μmol glucose. h^{-1}. g^{-1} nodule FW)	136.2±19.8	109.8±34.8
Fructokinase (μmol fructose-6-P. h^{-1}. g^{-1} nodule FW)	36.6±4.8	27.6±4.2

Table 4.6 The Effect of Nitrate Treatment on PEP Carboxylase Activity. Plants were grown as described in the legend to Fig. 4.1 except that the nitrate treatment period was 7 d and nodules were harvested in November, 47 DAP. Ten nodulated root systems, harvested randomly from three pots per treatment were assayed for nitrogenase activity using the standard acetylene reduction assay (see Chapter 2). Data are the mean ± SD (n=4), each replicate consisting of two plants. Nodules detached from the remaining plants were assayed for PEP carboxylase activity as described in Chapter 2. Data are the mean ± SD of four replicate nodule extracts.

Enzyme activity	control	treated
Nitrogenase (μmol C_2H_4. h^{-1}. g^{-1} nodule FW)	5.9±0.8	1.1±0.2
PEP Carboxylase (μmol PEP. h^{-1}. g^{-1} nodule FW)	264±18	150±12

CHAPTER 5

N_2 and Nitrate Assimilation in a Range of Soybean Genotypes

5.1 Introduction

Two major hypotheses have been proposed to explain the inhibitory effect of nitrate on nitrogenase activity. The first postulates competition between nitrate reductase in the leaves and nitrogenase in the nodules for available carbon skeletons and reducing equivalents, resulting in reduced allocation of photosynthate to the nodules (Oghoghorie and Pate 1971). The second postulates an interaction between nitrite, the initial product of nitrate reduction, and nitrogenase and/or leghaemoglobin, resulting in the inactivation of these proteins (Rigaud and Puppo 1977, Trinchant and Rigaud 1980). This chapter examines these hypotheses by comparing the effect of short-term (2 d), delayed nitrate treatment on N_2 fixation, nitrate uptake and NRA of five soybean genotypes differing from one another in their susceptibility to the inhibitory effect of nitrate on nodulation and N_2 fixation. N_2 fixation was estimated from the acetylene reduction activity of nodulated roots and the ureide concentration in the xylem sap of intact plants. Four soybean cultivars (Bragg, Hill, Leslie and Lee) and a mutant derivative of soybean cv. Bragg (nts382) were compared. Three of the four cultivars (Hill, Leslie and Lee) and nts382 had been previously selected for their improved ability to nodulate and fix N_2 when nitrate was present from the time of planting (Carroll et

al. 1985 a, b, Betts 1986). Bragg was included as a nitrate susceptible cultivar.

All of the above experiments were done in pots of either sand or a sand/vermiculite mixture. In addition, a comparison of the effect of delayed nitrate treatment on nitrogenase (acetylene reduction) activity of a wider range of soybean genotypes was carried out in hydroponic culture.

5.2 Results

5.2.1 Nitrogenase Activity

Short-term (2 d), delayed nitrate treatment inhibited nitrogenase activity (expressed on a nodule fresh weight basis) of Bragg, nts382, Hill, Leslie and Lee by 69%, 51%, 74%, 76% and 47%, respectively (Fig. 5.1). Thus, nts382 and Lee were less affected than the other genotypes. These genotypes were also less susceptible to the inhibitory effect of nitrate treatment on nodule development (Carroll et al. 1985 a, b, Betts 1986). Hill and Leslie, on the other hand, which had previously been selected for their improved ability to nodulate and fix N_2 in the presence of nitrate (Betts 1986), were equally as susceptible to the inhibitory effect of delayed nitrate treatment as the nitrate susceptible cultivar Bragg.

In Fig. 5.2 the data presented in Fig. 5.1 are expressed on a per plant basis rather than a nodule fresh weight basis. The magnitude of

the effect of nitrate treatment, expressed as a percentage of the control value, was similar to that seen when nitrogenase activity was expressed on a nodule fresh weight basis (Fig. 5.1). Nitrogenase activity, both per g nodule fresh weight and per plant, was very low for nts382 when compared with the other genotypes. This will be discussed further in Chapter 6.

5.2.2 Xylem Sap Ureide Content

Short-term (2 d), delayed nitrate treatment reduced the concentration of ureides in the xylem sap of Bragg, nts382, Hill, Leslie and Lee by 67%, 40%, 77%, 75% and 44%, respectively (Fig. 5.3). Thus, the proportional decline in the xylem sap ureide concentration, in response to delayed nitrate treatment, was similar to that observed for nitrogenase activity. Interestingly, however, whilst nitrogenase activity per plant of nts382 was less than half of that of the other genotypes the concentration of ureides in the xylem sap of the mutant was almost two-fold greater. This is in conflict with the generally accepted conclusion that ureides are synthesized predominantly as the products of N_2 fixation in soybean (see Schubert and Boland 1984). It is possible that the unexpectedly high concentration of ureides in the xylem sap of nts382 may simply be due to a slower rate of exudation of xylem sap in the mutant. This will be discussed in detail in Chapter 6.

5.2.3 Plant and Nodule Growth

There was no significant effect of delayed nitrate treatment on nodule fresh weight (Table 5.1). Neither was they any significant difference between the genotypes in nodule fresh weight except for nts382 which had approximately twice the nodule fresh weight of the other genotypes. This confirmed earlier observations regarding the nodulation phenotype of the mutant (Carroll et al. 1985 a, b).

All of the genotypes, except nts382, had comparable shoot and root (minus nodules) dry weights (Table 5.2). The dry weight of nts382 shoots and roots (minus nodules) was less than half of that of the other genotypes. This might be explained by the lower whole plant nitrogenase activity of the mutant. However, the xylem sap ureide content of nts382 was apparently higher than that of Bragg (see above) and total organic nitrogen content of the mutant is also higher than that of Bragg (Day et al. 1986). Therefore, nitrogen availability was probably not limiting the growth of nts382.

In other studies, whole plant nitrogenase activity of nts382 was similar to that of Bragg, but shoot and root growth of the mutant was still reduced (Carroll et al. 1985 a, b; Day et al. 1986). These authors attributed the reduced shoot and root growth of nts382 to its prolific nodulation. Increased root respiration and/or alterations in phytohormone production or action, in the mutant, may also be involved (Day et al. 1986, Gresshoff and Delves 1986, in press).

5.2.4 Nitrate Uptake and Leaf Nitrate Reductase Activity

Table 5.3 shows a comparison of the xylem sap nitrate content of the five soybean genotypes. Although nitrogenase activity of nts382 and Lee was much less susceptible to nitrate-induced inhibition (Fig. 5.1), the concentration of nitrate in the xylem sap of these genotypes was not significantly different from that of the nitrate susceptible cultivar, Bragg. Furthermore, although the xylem sap nitrate concentration of Hill was significantly greater than that of the other genotypes, nitrogenase activity of this genotype was not more susceptible to nitrate induced-inhibition.

Fig. 5.4 illustrates a comparison of the *in vivo* activity of nitrate reductase in the youngest fully expanded trifoliolate of the five genotypes. Low levels of nitrate reductase activity (NRA), assayed in the presence of exogenous nitrate, were detected in the leaves of control plants of all genotypes. Following nitrate treatment there was a marked increase in NRA, assayed either in the presence or absence of added nitrate, indicating the presence of an inducible nitrate reductase (Streit *et al*. 1985).

NRA assayed in the absence of exogenous nitrate gives an indication of the availability of nitrate to nitrate reductase *in situ* and therefore an indication of NRA *in situ*. The level of NRA observed with the plus nitrate assay was not significantly higher than that observed with the minus nitrate assay indicating that nitrate uptake was probably not limiting NRA *in situ*. NRA of nts382 and Lee, assayed in the absence

of exogenous nitrate, was not significantly less than that of Bragg and Leslie even though the former two genotypes were less susceptible to nitrate-induced inhibition of nitrogenase activity. Furthermore, Hill, which had the lowest NRA of all was amongst the more nitrate susceptible genotypes.

5.2.5. Broader Screen of Soybean Genotypes for Reduced Susceptibility of Nitrogenase Activity to Inhibition by Nitrate.

A broad screen of soybean genotypes for reduced susceptibility of nitrogenase activity to inhibition by nitrate was carried out in hydroponic culture. The results are shown in Table 5.4. The amount of variation between the genotypes, in relation to the magnitude of the inhibitory effect of short-term (2 d) delayed nitrate treatment on nitrogenase activity, was not very great. The percentage inhibition ranged from 22%-60%. However, most (72%) of the genotypes were clustered between 40%-60% inhibition. There was no significant effect of nitrate treatment on nodule fresh weight of any of the genotypes (Table 5.5).

As in the sand/vermiculite culture experiment (Fig. 5.1), nts382 and Lee were amongst the least susceptible genotypes and Bragg was amongst the most susceptible genotypes. The placement of Hill and Leslie amongst the least susceptible genotypes in the hydroponics experiment was in agreement with the results of Betts (1986) for nitrate-susceptibility of nodulation but it was in conflict with the results obtained in the sand/vermiculite culture experiment (Fig. 5.1).

The nitrogenase activities obtained in the hydroponics experiment were approximately three-fold higher than those obtained in the sand/vermiculite culture experiment (compare Table 5.4 and Fig. 5.1). The difference is most probably due to the different growth conditions.

5.3 Discussion

In hydroponic culture, the soybean cultivars, Hill, Leslie and Lee, and the mutant, nts382, which had been previously selected for their improved ability to nodulate and fix N_2 in the presence of high levels of nitrate (Carroll et al. 1985 a, b; Betts 1986), also showed reduced susceptibility to the inhibitory effect of short-term (2 d), delayed nitrate treatment on N_2 fixation. In sand/vermiculite culture, on the other hand, only Lee and nts382 showed reduced nitrate-susceptibility of N_2 fixation. The differences between the results of the hydroponics experiment and the sand/vermiculite experiment may be due to differences in the level of nitrate available to the plants. This is indicated by the observation that all genotypes were more strongly inhibited in the sand/vermiculite culture experiment than they were in the hydroponics experiment. Due to this, any minor differences in nitrate susceptibility may have been overridden in the sand/vermiculite culture experiment.

Some of the cultivars tested for the effect of delayed nitrate treatment on nitrogenase activity in the hydroponics experiment, have also been tested for the effect of continuous nitrate treatment on nitrogenase activity in the same hydroponics culture system (Gibson and Harper 1985). Elf, Avoyelles and Lincoln were the three cultivars least

susceptible to the inhibitory effect of continuous nitrate treatment on nitrogenase activity (Gibson and Harper 1985). In the present study, Lincoln was amongst the cultivars least susceptible to the inhibitory effect of delayed nitrate treatment but Avoyelles and Elf were amongst the more susceptible cultivars. Scott, which was one of the less susceptible cultivars in the present study, was one of the more susceptible cultivars in the study of Gibson and Harper (1985). These results suggest that, reduced susceptibility of N_2 fixation to the inhibitory effect of continuous nitrate treatment may be a result of reduced nitrate susceptibility of nodulation rather than of N_2 fixation per se. Furthermore, differences between the effects of nitrate on nodulation and N_2 fixation suggest that the mechanisms involved in the inhibitory effect of nitrate on these parameters may differ from one another.

There was no correlation between the level of NRA in the leaves and the level of inhibition of N_2 fixation in the five genotypes selected for more intensive study. This suggests that competition between nitrate reductase and nitrogenase for available energy sources may not be involved in precipitating the nitrate-induced decline in nitrogenase activity in soybean. In agreement with this conclusion, Streeter (1981) reported an 80% decline in nitrogenase activity of soybean, in response to delayed nitrate treatment, with no effect on total nodule carbohydrate content. Similarly, nodule carbohydrate content and the allocation of ^{14}C-labelled photosynthate to the nodules did not decline between 6 and 24 hours after nitrate application even though nitrogenase activity was progressively inhibited during this time in soybean (Wasfi

and Prioul 1986). These results are apparently in conflict with those of earlier studies in which it was found that nitrate treatment reduced the allocation of photosynthate to the nodules of pea, subclover and soybean (Small and Leonard 1969, Latimore et al. 1977). However, the nitrate treatment period in these studies was much longer and therefore the reduced allocation of photosynthate to the nodules may have been a result, rather than the cause, of the inhibition of nitrogenase activity.

In conclusion, the results of the present study indicate that soybean genotypes selected for reduced susceptibility of nodulation to nitrate-inhibition may also show reduced susceptibility of nitrogenase activity to nitrate-inhibition. However, this is not always the case, indicating that the mechanisms involved in the inhibitory effect of nitrate may differ between nodulation and N_2 fixation. The absence of any correlation between the level of NRA and the magnitude of the inhibitory effect of nitrate on nitrogenase activity suggests that competition between nitrate reductase and nitrogenase for available energy sources may not be involved in the inhibitory effect of nitrate on nitrogenase activity in soybean.

Fig. 5.1 The Effect of Nitrate Treatment on Nitrogenase Activity per g Nodule Fresh Weight of Five Soybean Genotypes. The five genotypes inoculated with B. japonicum strain USDA 110 were grown in the absence of combined nitrogen in 8 l pots of vermiculite (10 plants/pot) in a naturally-illuminated glasshouse during January/February, as described in Chapter 2. Nitrate treatment was commenced 39 DAP with control plants receiving 10 mM KCl and treated 10 KNO_3 for 2 consecutive days prior to harvest. Four plants from one pot per treatment were assayed for nitrogenase activity using the standard acetylene reduction assay (see Chapter 2). Data are the mean ± SD (n=4).

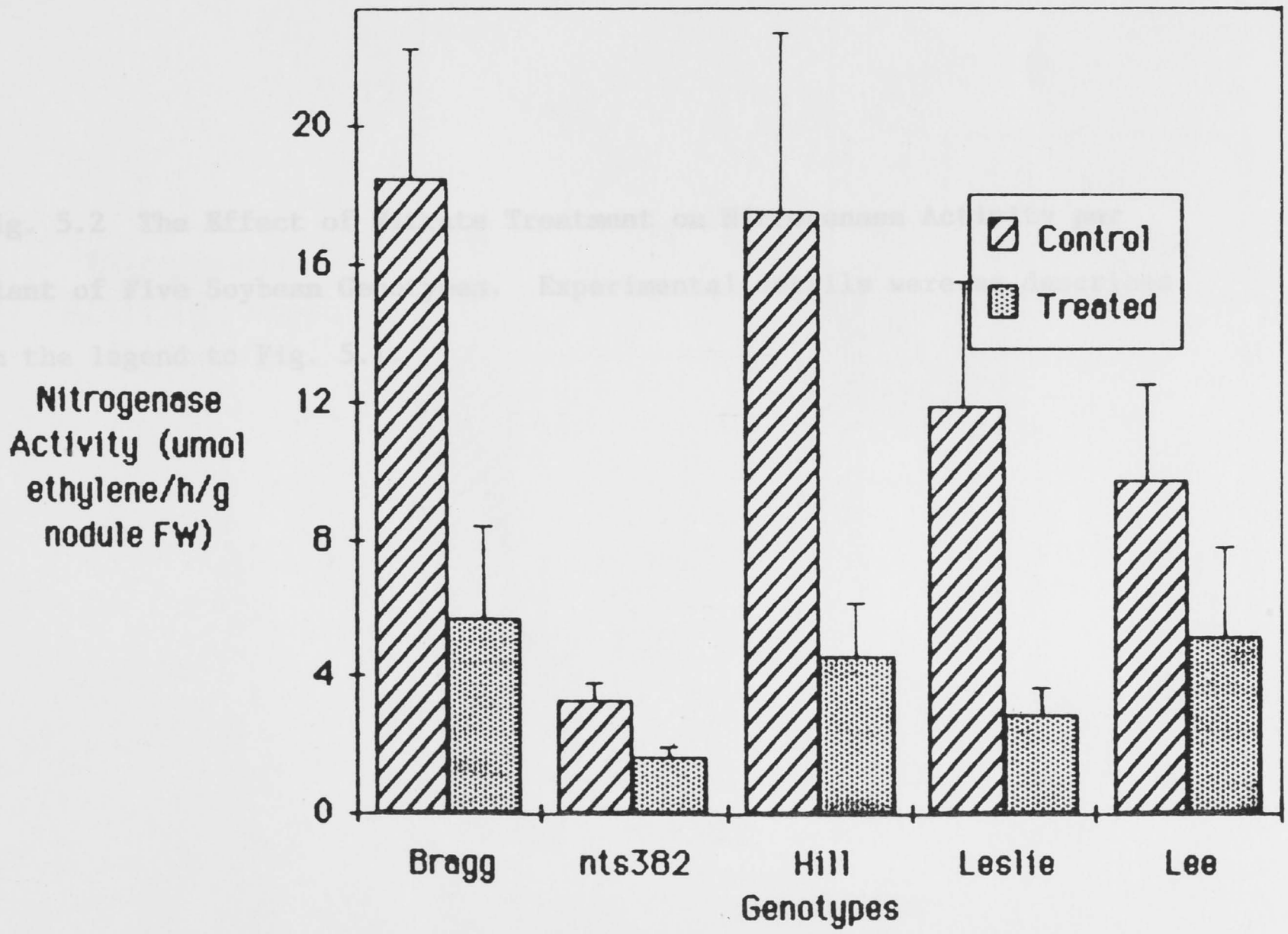

Nitrogenase Activity (umol ethylene/h/g nodule FW)
0
4
8
12
16
20
Control
Treated
Bragg
nts382
Hill
Leslie
Lee
Genotypes

Fig. 5.2 The Effect of Nitrate Treatment on Nitrogenase Activity per Plant of Five Soybean Genotypes. Experimental details were as described in the legend to Fig. 5.1.

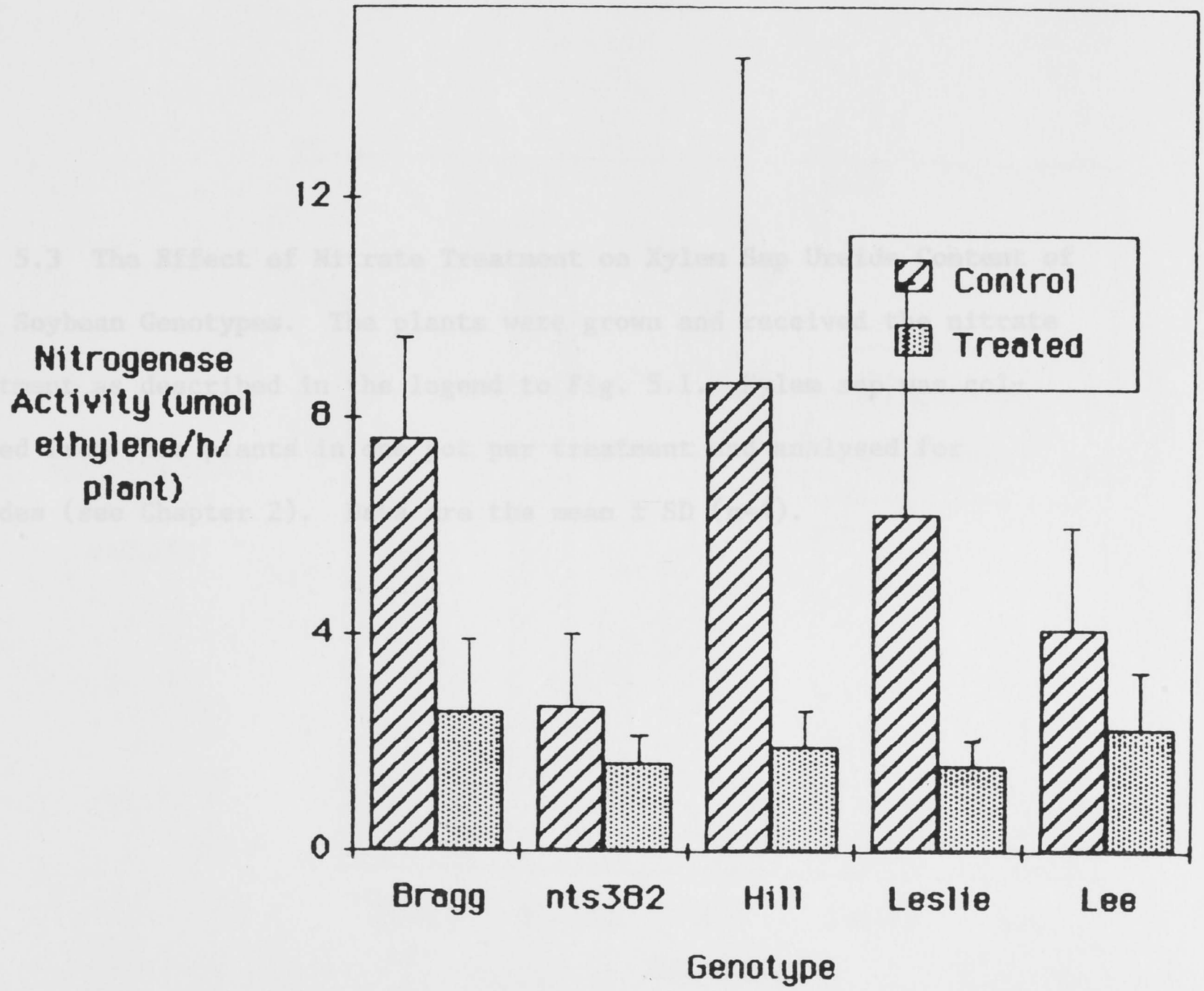

Nitrogenase Activity (umol ethylene/h/ plant)
12
8
4
0
Control
Treated
Bragg
nts382
Hill
Leslie
Lee
Genotype

Fig. 5.3 The Effect of Nitrate Treatment on Xylem Sap Ureide Content of Five Soybean Genotypes. The plants were grown and received the nitrate treatment as described in the legend to Fig. 5.1. Xylem sap was collected from four plants in one pot per treatment and analysed for ureides (see Chapter 2). Data are the mean ± SD (n=4).

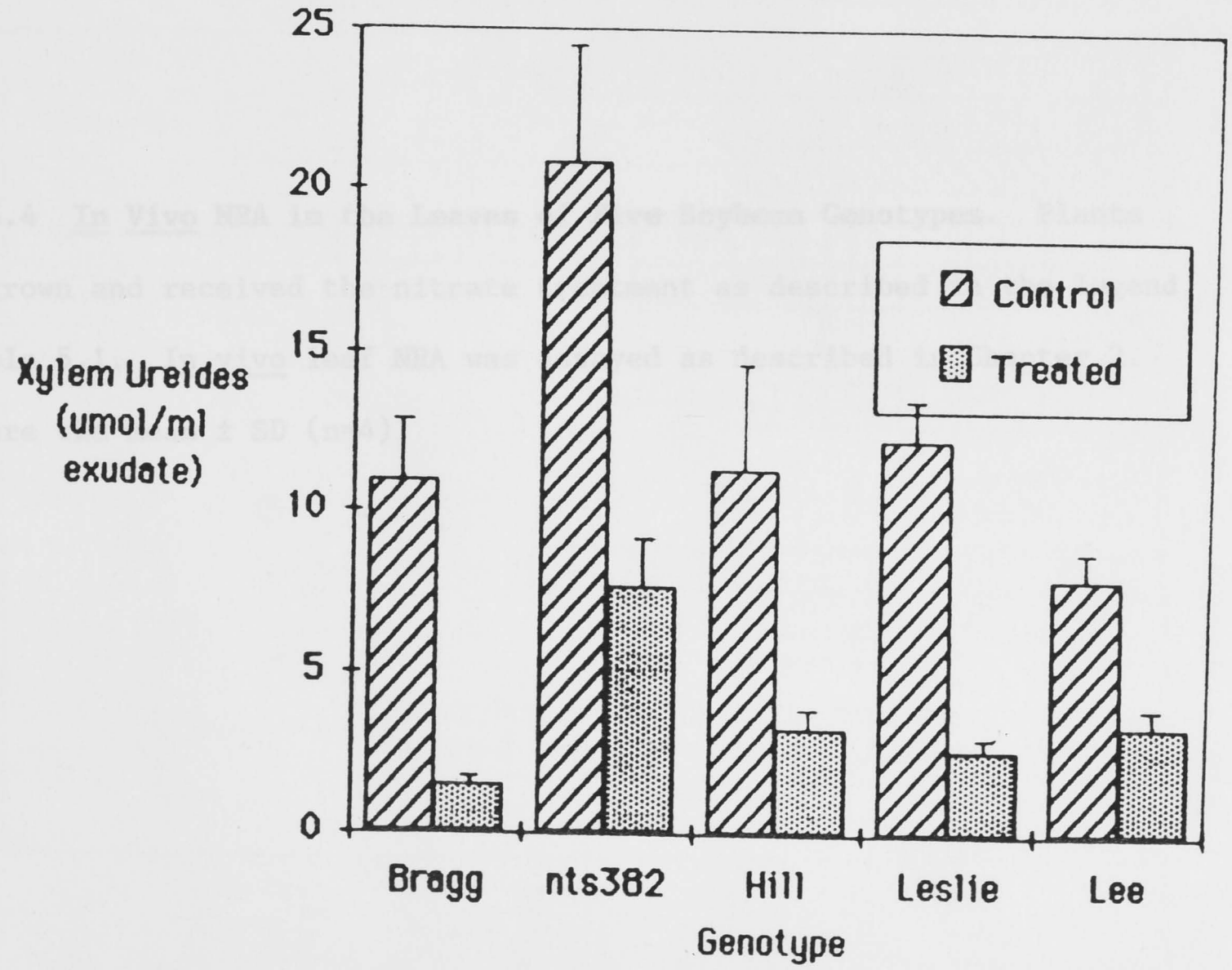

25
20
15
10
5
0
Xylem Ureides
(umol/ml
exudate)
Control
Treated
Bragg
nts382
Hill
Leslie
Lee
Genotype

Fig. 5.4 <u>In Vivo</u> NRA in the Leaves of Five Soybean Genotypes. Plants were grown and received the nitrate treatment as described in the legend to Table 5.1. <u>In vivo</u> leaf NRA was assayed as described in Chapter 2. Data are the mean ± SD (n=4).

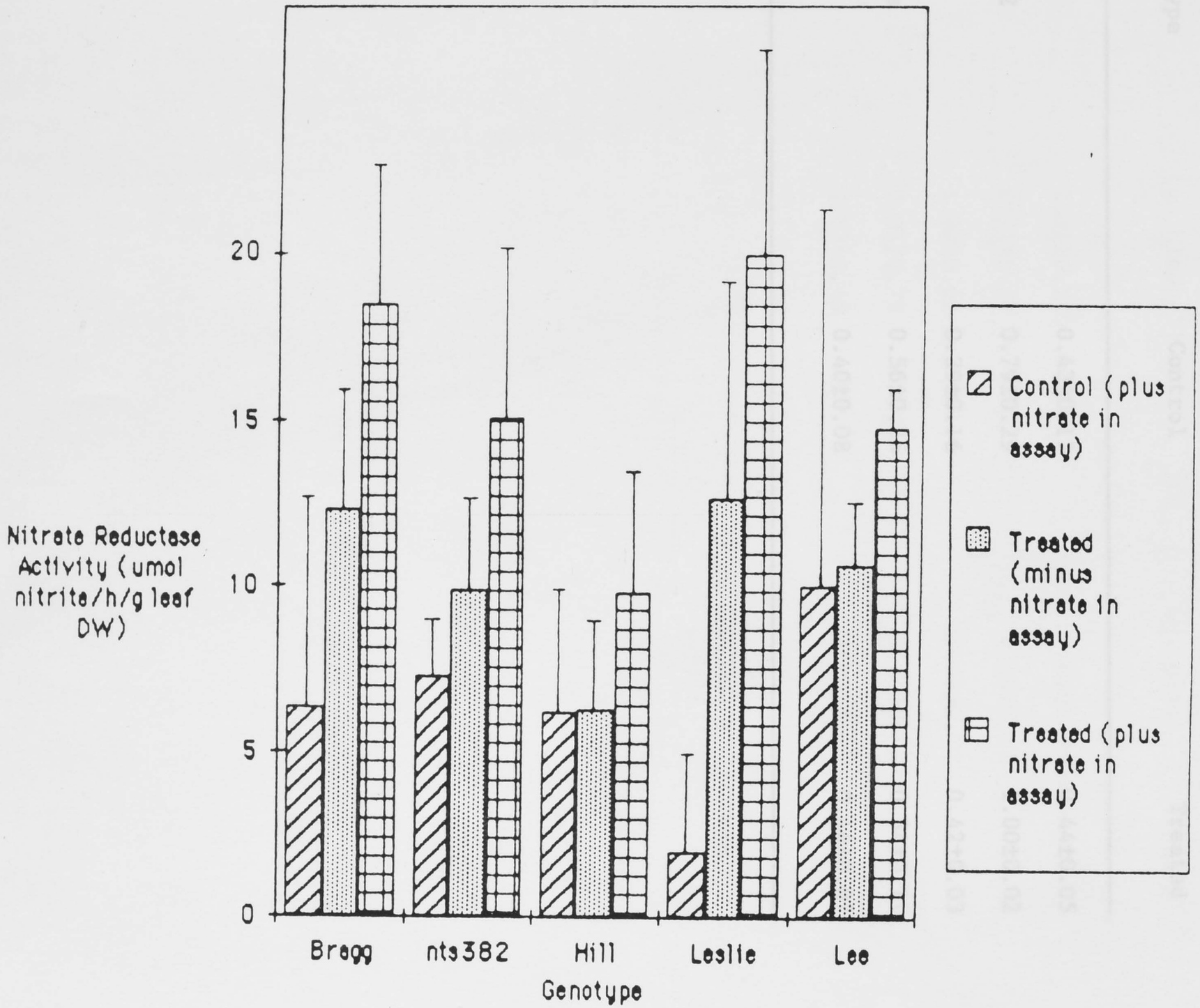

20
15
10
5
0
Nitrate Reductase Activity (umol nitrite/h/g leaf DW)
Bregg
nts382
Hill
Leslie
Lee
Genotype
Control (plus nitrate in assay)
Treated (minus nitrate in assay)
Treated (plus nitrate in assay)

Table 5.1 Nodule Fresh Weight of the Five Soybean Genotypes. Plants were grown and received the nitrate treatment as described in the legend to Fig. 5.1. Data are the mean ± SD (n=4).

Genotype	Nodule Fresh Weight (g. plant^{-1}) Control	Treated
Bragg	0.43±0.13	0.44±0.05
nts382	0.79±0.29	1.00±0.02
Hill	0.39±0.16	0.42±0.03
Leslie	0.56±0.35	0.54±0.11
Lee	0.40±0.08	0.46±0.07

Table 5.2 Plant Dry Weight of the Five Soybean Genotypes. Plants were grown, in the absence of combined nitrogen, as described in the legend to Fig. 5.1. Data are the mean ± SD (n=6).

Genotype	Shoot Dry Weight ($g.\ plant^{-1}$)	Root (minus nodules) Dry Weight ($g.\ plant^{-1}$)
Bragg	1.91±0.17	0.62±0.06
nts 382	0.74±0.05	0.36±0.03
Hill	1.89±0.60	0.69±0.22
Leslie	2.01±0.79	0.84±0.35
Lee	2.12±0.48	0.84±0.17

Table 5.3 Xylem Sap Nitrate Content of the Five Soybean Genotypes.

Plants were grown and received the nitrate treatment as described in the legend to Fig. 5.1. Xylem sap was collected from four plants in one pot per genotype and analysed for nitrate as described in Chapter 2. Data are the mean ± SD (n=4).

Genotype	Xylem nitrate content ($\mu mol.\ ml^{-1}$ xylem sap)
Bragg	8.5±0.3
nts382	7.5±0.5
Hill	13.5±1.2
Leslie	8.6±1.7
Lee	7.7±0.3

Genotype	Nitrogenase Activity (μmol C_2H_4. h^{-1}. g^{-1} nodule FW) Control	Treated	% Inhibition
nts382	16.7±2.2	13.0±0.8	22
Leslie	13.1±2.7	9.9±1.3	24
Lincoln	13.5±3.0	9.6±3.6	29
Hill	18.9±4.5	12.8±2.9	32
Scott	20.5±3.1	13.3±2.1	36
Williams	14.2±2.2	8.0±1.5	44
Clark '63	15.3±4.7	8.4±3.2	45
Lee '74	20.0±5.2	10.9±2.3	46
Fitzroy	19.4±5.4	10.1±1.5	48
Semstar	24.3±3.9	12.5±2.8	48
SU 408	19.7±4.3	9.9±1.3	50
Dixie	24.9±5.1	12.0±5.3	52
Avoyelles	18.3±6.1	8.7±3.8	53
Elf	16.5±4.0	7.5±2.4	55
Crawford	19.4±4.5	8.7±1.6	55
Bragg	30.4±4.5	13.4±2.7	56
Harosoy	18.9±7.3	7.4±1.8	60
D67-4823	22.6±5.8	9.1±2.0	60

Table 5.4 The Effect of Short-Term, Delayed Nitrate Treatment on Nitrogenase Activity of a Range of Soybean Genotypes. Plants were grown hydroponically as described in section 2.2.2. Data are the mean ± SD

(n=4). This experiment was carried out in collaboration with Dr. A.H. Gibson, Division of Plant Industry, CSIRO, Canberra, ACT, Australia.

Table 5.5 The Effect of Short-Term, Delayed Nitrate Treatment on Nodule Fresh Weight of a Range of Soybean Genotypes. Experimental details were as described in the legend to Table 5.4.

Genotype	Nodule Fresh Weight (g. plant^{-1}) Control	Treated
nts 382	0.94±0.17	0.94±0.30
Leslie	0.68±0.18	0.70±0.11
Lincoln	0.88±0.10	0.82±0.15
Hill	0.53±0.09	0.44±0.07
Scott	0.39±0.15	0.27±0.08
Williams	0.60±0.20	0.60±0.11
Clark '63	0.50±0.06	0.53±0.12
Lee '74	0.55±0.13	0.60±0.10
Fitzroy	0.53±0.12	0.54±0.14
Semstar	0.69±0.08	0.60±0.11
SU 408	0.71±0.08	0.69±0.16
Dixie	0.39±0.17	0.38±0.05
Avoyelles	0.38±0.16	0.31±0.11
Elf	0.72±0.12	0.67±0.13
Crawford	0.69±0.14	0.73±0.07
Bragg	0.65±0.13	0.59±0.14
Harosoy	0.52±0.12	0.56±0.08
D67-4823	0.96±0.17	0.75±0.16

CHAPTER 6

Nitrogen Fixation and Ureide Metabolism in Soybean cv. Bragg and its Mutant Derivative nts382

6.1 Introduction

The isolation and preliminary characterisation of the super-nodulating soybean mutant nts382 have been described (Carroll et al. 1985 a, b). This mutant, which was derived from soybean cv. Bragg, was isolated on the basis of its ability to nodulate in the presence of high levels of nitrate. In Chapter 5 it was shown that, when compared with Bragg, nts382 was less susceptible to the inhibitory effect of delayed nitrate treatment on N_2 fixation. This treatment separates the effect of nitrate on N_2 fixation per se from its effect on nodulation. In this chapter, the observations presented in Chapter 5 are confirmed and extended. Ureide biosynthesis and utilization in Bragg and nts382 are examined and the nodule cytoplasmic protein profiles of the mutant and its parent cultivar are compared. The aim of the latter was to identify proteins which may be associated with the altered symbiotic phenotype of the mutant.

6.2 Results

6.2.1 Nitrogenase Activity and Nodule Fresh Weight

Bragg and nts382 were compared with one another on the basis of their nitrogenase activity, nodule fresh weight and the effect of delayed nitrate treatment on these parameters. The results are shown in

Table 6.1. Nitrogenase activity, per g nodule fresh weight, of control nts382 plants was three-fold less than that of control Bragg plants whereas nodule fresh weight was two-fold greater. In contrast, whole plant nitrogenase activity was not significantly different between control Bragg and nts382 plants.

Short-term (2 d), delayed nitrate treatment inhibited nitrogenase activity, per g nodule fresh weight, of Bragg by 75%. The inhibitory effect on nitrogenase activity of nts382 was not so marked, however, being only 51%. Almost identical results were obtained when nitrogenase activity was expressed on a per plant basis. Nodule fresh weight was not affected by delayed nitrate treatment.

6.2.2 Ureide Content of the Xylem Sap and Nodules

The ureide content of the xylem sap and nodules of Bragg and nts382 and the effects of delayed nitrate treatment on these parameters are shown in Table 6.2. Xylem sap ureide content of control nts382 plants was two-fold greater than that of control Bragg plants. This was consistent with the nodule fresh weight of nts382 being two-fold greater than that of Bragg (Table 6.1). However, it was in conflict with the observation that whole plant nitrogenase activity was no greater for control Bragg plants than it was for control nts382 plants (Table 6.1).

Short-term (2 d), delayed nitrate treatment reduced the ureide content of the xylem sap of Bragg and nts382 by 62% and 41%, respectively. The lesser effect of nitrate treatment on xylem sap ureide content of nts382 was consistent with the lesser effect on nitrogenase activity of the mutant (Table 6.1). The effect of delayed nitrate treatment on xylem sap ureide content was proportionally similar to,

although slightly less than, the effect on nitrogenase activity, for both Bragg and nts382.

Since nitrogenase activity, per g nodule fresh weight, of control nts382 plants was less than that of treated Bragg plants, it could be argued that any effect of nitrate treatment on nts382 is likely to appear less pronounced. However, the ureide content of control nts382 plants was two-fold higher than that of control Bragg plants and the effect of nitrate treatment on this parameter was also less marked for nts382 than it was for Bragg.

There was no effect of short-term (2 d), delayed nitrate treatment on the ureide content of the nodules of either Bragg or nts382, when expressed on a nodule fresh weight basis. However, like the ureide content of the xylem sap, nodule ureide content of nts382 was several-fold greater than that of Bragg. The difference was in the order of four-fold. This contrasted with the observation that nitrogenase activity, per g nodule fresh weight, of control nts382 plants was four-fold less than that of control Bragg plants (Table 6.1).

The nitrate content of the xylem sap of treated plants was similar for Bragg and nts382 (Table 6.2), indicating that the relative nitrate tolerance of the mutant was not due to decreased nitrate uptake (see also Chapter 5).

Ureides are found in the xylem sap of non-nodulated plants, albeit at low levels compared with effectively nodulated plants (McClure and Israel 1979, Streeter 1979). Furthermore, soybean roots can synthesize ureides via de novo purine synthesis (Polayes and Schubert 1984). Therefore, one possible explanation for the high level of ureides in nts382 may be elevated synthesis in the roots. However, there was no

difference between four-week old Bragg and nts382 plants, grown uninoculated in the presence of nitrate (10 mM KNO_3), in the concentration of ureides in the xylem sap. Xylem sap ureide concentration was equally low in both, being 0.21 ± 0.03 and 0.29 ± 0.05 µmol. ml^{-1} exudate (mean ± SD, n=4) for Bragg and nts382, respectively.

6.2.3 Enzymes of Carbon Metabolism in the Nodules

In section 6.2.1 it was noted that nitrogenase activity, per g nodule fresh weight, of nts382 was several-fold less than that of Bragg (see also Chapter 5; Carroll et al. 1985 a, b; Day et al. 1986). One explanation for this may be that the metabolism of sucrose in the plant fraction of nts382 nodules, to yield carboxylic acids for respiration by the bacteroids, is impaired. Sucrose is the form in which photosynthate is imported by legume nodules (Bach et al. 1958) and carboxylic acids are most probably the respiratory substrates utilized by bacteroids (see Ronson and Astwood 1985). Table 6.3 shows a comparison of alkaline invertase, fructokinase and PEP carboxylase activities of Bragg and nts382 nodules. Alkaline invertase and fructokinase are the major enzymes involved in the initial degradation of sucrose in developing soybean nodules (Morell and Copeland 1984). PEP carboxylase is involved in the synthesis of carboxylic acids which can act either as carbon skeletons for the assimilation of fixed nitrogen or as respiratory substrates for the bacteroids (see Gadal 1983). Although nitrogenase activity of nts382 nodules was 69% less than that of Bragg nodules (Table 6.1), invertase activity was only 33% less, fructokinase activity was not significantly different and PEP carboxylase activity was only 21% less. Therefore, it is unlikely that the potential capacity for

sucrose degradation and carboxylic acid biosynthesis in the nodules is limiting nitrogenase activity of nts382. Furthermore, since the in vivo activity of alkaline invertase is regulated mainly by the availability of sucrose (Morell and Copeland 1984), it is unlikely that the rate of photosynthate flux to the nodules is limiting nitrogenase activity of nts382 either. However, a thorough analysis of photosynthate flux to the nodules including an examination of ^{14}C-incorporation into the nodules from ^{14}C-labelled CO_2 is required to determine this.

6.2.4 Bacteroid Protein and Leghaemoglobin Content of the Nodules

Table 6.4 shows a comparison of the bacteroid protein and leghaemoglobin content of Bragg and nts382 nodules. The bacteroid protein content of nts382 nodules, expressed on a nodule fresh weight basis, was 2.5-fold less than that of Bragg nodules. This may, at least partially, explain the four-fold lower nitrogenase activity, per g nodule fresh weight, of nts382. The leghaemoglobin content of nts382 nodules, as indicated by the haem level expressed on a nodule fresh weight basis, was also less than that of Bragg nodules. The difference was of the order of two-fold. Since bacteroids are responsible for the synthesis of the haem moiety of leghaemoglobin (see Appleby 1984), the low bacteroid protein content of nts382 nodules may explain their low haem content. In view of this, it would be interesting to determine whether synthesis of the globin moiety of leghaemoglobin is also decreased. This has been shown to be a plant gene product (see Appleby 1984).

6.2.5 Enzymes of Purine Oxidation in the Nodules

In section 6.2.2 it was noted that the ureide content of the xylem sap and nodules of nts382 was higher than that of Bragg. This indicates that the capacity for ureide biosynthesis may be elevated in nts382 nodules and/or roots. Ureide biosynthesis, in soybean nodules, proceeds via the de novo synthesis of purines, followed by the oxidative degradation of those purines to yield ureides (see Schubert and Boland 1984). Table 6.5 shows a comparison of the in vitro activities of xanthine dehydrogenase (XDH), uricase and allantoinase in the nodules of Bragg and nts382. These enzymes catalyse the oxidative degradation of purines to ureides. An analysis of purine biosynthesis was beyond the scope of the present study. Substantial activities of all three enzymes were detected but there were no significant differences between the mutant and its parent cultivar in the level of activity of any of the enzymes.

6.2.6 Allantoin and Allantoate Degradation in the Leaves

The level of ureides in the leaves of nts382 was three-fold higher than it was in Bragg leaves (Table 6.6). Therefore, the high ureide content of nts382 may be due to an impairment in ureide degradation in the leaves. Allantoate accounted for 83% and 72% of the ureides in the leaves of Bragg and nts382, respectively. This suggests that allantoate hydrolysis may be the rate limiting step in ureide utilization. The latter is consistent with the conclusion of Thomas and Schrader (1981 a). It should be noted, however, that allantoate constituted 67% and 85% of the ureides in the xylem sap of Bragg and nts382, respectively. Therefore, the high allantoate/allantoin ratio of the leaves may simply

be a reflection of the ratio of these compounds in the xylem sap and may not necessarily indicate that allantoate hydrolysis is rate limiting. Table 6.7 shows a comparison of allantoinase activity and allantoate hydrolysis in the leaves of Bragg and nts382. Allantoinase was assayed as allantoate production from allantoin and allantoate hydrolysis as glyoxylate production from allantoin. The in vivo rate of glyoxylate production from allantoin was only 1% of the in vivo activity of allantoinase. However, there were no differences between Bragg and nts382 in either allantoinase activity or the rate of allantoate hydrolysis.

6.2.7 Polyacrylamide Gel Analysis of Nodule Cytoplasmic Proteins

Extracts of the nodule cytoplasmic proteins of Bragg and nts382 were analysed by polyacrylamide gel electrophoresis (PAGE). Plants received either 10 mM KCl (control) or 10 mM KNO_3 (treated) for 7 d prior to harvest (see Legend to Fig. 6.1). One dimensional SDS-PAGE revealed no differences either between Bragg and nts382 nodules or between control and nitrate treated nodules (Fig. 6.1). However, when the resolution was improved by using two dimensional PAGE, a protein which was apparently more abundant in nts382 nodules than it was in Bragg nodules was detected (compare Figs. 6.2A and 6.2B). This protein focused near the acidic end of the first dimension IEF gel and had a molecular weight of approximately 24Kd. Although other quantitative differences are obvious between the two dimensional gels, only the difference in the 24Kd protein was constantly reproducible. There were no detectable differences between control and nitrate treated nodules even using two dimensional PAGE (not shown).

6.3 Discussion

When compared with its parent cultivar Bragg, nitrogenase activity, as determined by acetylene reduction, of the supernodulating, nitrate-tolerant soybean mutant nts382 was less susceptible to the inhibitory effect of delayed nitrate treatment (this chapter, see also Chapter 5). A similar result was obtained when N_2 fixation was estimated from the concentration of ureides in the xylem sap. The relative nitrate-tolerance of nts382 could not be explained either by reduced nitrate uptake (this chapter, see also Chapter 5) or by a lower level of nitrate reductase activity in the leaves (see Chapter 5).

Nitrogenase activity, per g nodule fresh weight, of nts382, was almost four-fold less than that of Bragg. However, extractable activity of alkaline invertase, fructokinase and PEP carboxylase, expressed on the same basis, did not differ between the mutant and its parent cultivar. Therefore, the low nitrogenase activity of nts382 could not be explained by reduced activity of these enzymes and any effect such a reduction may have had on the supply of respiratory substrates to the bacteroids. In contrast, the bacteroid protein content of nts382 nodules, expressed on a nodule fresh weight basis, was 2.5-fold less than that of Bragg nodules and this may, at least partially, explain the low nitrogenase activity, per g nodule fresh weight, of nts382.

Although nitrogenase activity, per plant, of nts382 was no greater than that of Bragg, the concentration of ureides in the xylem sap of the mutant was several-fold higher. This is in conflict with the generally accepted conclusion that ureide biosynthesis is virtually uniquely coupled to N_2 fixation in soybean (see Schubert and Boland 1984). The high concentration of ureides in the xylem sap of nts382 could not be

explained by the slower (relative to Bragg) xylem sap exudation rate generally observed for the mutant, because the ureide content of the leaves was also high. Neither could it be explained by an impairment in nitrogen utilization in the leaves since (1) the degradation of ureides to yield glyoxylate, in the leaves of nts382, was apparently unimpaired (this study), (2) urea and nitrate stimulate the growth of symbiotically-grown nts382 plants (Carroll et al. 1985 b) and (3) nitrate-dependent nts382 plants grow just as well as nitrate-dependent Bragg plants (Day et al. 1986). Interestingly, the Kjeldahl nitrogen content of N_2-dependent nts382 plants is twice that of N_2-dependent Bragg plants whereas the Kjeldahl nitrogen content of nitrate-dependent plants is similar for Bragg and nts382 (Day et al. 1986). This parallels differences in ureide content of the mutant and its parent cultivar.

The pathway of ureide utilization in soybean leaves commences with the allantoinase catalysed conversion of allantoin to allantoate (see Thomas and Schrader 1981 b). This is followed by the hydrolysis of allantoate to yield either ureidoglycolate and urea (Shelp and Ireland 1985) or ureidoglycine, ammonia and CO_2 (Winkler et al. 1985). The first of these alternatives is catalysed by allantoicase and the second by allantoate amidohydrolase. Recent evidence favours the allantoate amidohydrolase catalysed reaction (Winkler et al. 1985). Regardless of which of these enzymes is involved, the ultimate product of allantoate hydrolysis is most likely glyoxylate. In the present study, the in vivo rate of glyoxylate production from allantoin was only 1% of the in vivo activity of allantoinase. This was consistent with allantoate hydrolysis being rate limiting (Thomas and Schrader, 1981 a). However,

there were no differences between Bragg and nts382 in either allantoinase activity or the rate of allantoate hydrolysis. Therefore, it is unlikely that an impairment in ureide degradation in the leaves of nts382 is the explanation for the high ureide content of the mutant.

Another possible explanation for the high ureide content of the xylem sap and leaves of nts382 may be elevated ureide biosynthesis in the roots. Contrary to this, however, the level of ureides in the xylem sap of non-nodulated, nitrate-grown nts382 plants was the same as it was for Bragg plants. Nevertheless, this does not preclude the possibility of elevated ureide biosynthesis in either the roots or nodules of symbiotically-grown nts382 plants.

Nodule ureide content of nts382 was several-fold higher than that of Bragg. Given that xylem sap and leaf ureide content were also higher in the mutant, it is unlikely that the high level of ureides in the nodules can be explained by reduced export from the nodules.

If indeed the level of ureide biosynthesis is elevated in nts382 nodules, as the high level of ureides in the mutant suggests, then the extractable activities of the enzymes involved in ureide biosynthesis might also be expected to be high. Contrary to this, however, there were no differences between Bragg and nts382 nodules in the levels of extractable activity of the enzymes involved in the oxidative degradation of purines to ureides. This does not, however, preclude the possibility that purine biosynthetic activity is elevated in nts382 nodules and that it is this rather than purine oxidation which limits ureide biosynthesis.

Silver-stained, two dimensional polyacrylamide gels of bacteroid-free nodule extracts revealed the presence of a protein, with a

molecular weight of approximately 24Kd, which was more abundant in nts382 nodules than it was in Bragg nodules. A soybean nodulin located in the peribacteroid membrane has a molecular weight of 24Kd (Blumwald et al. 1985, Fortin et al. 1985). This may correspond to the protein which was amplified in nts382 nodules.

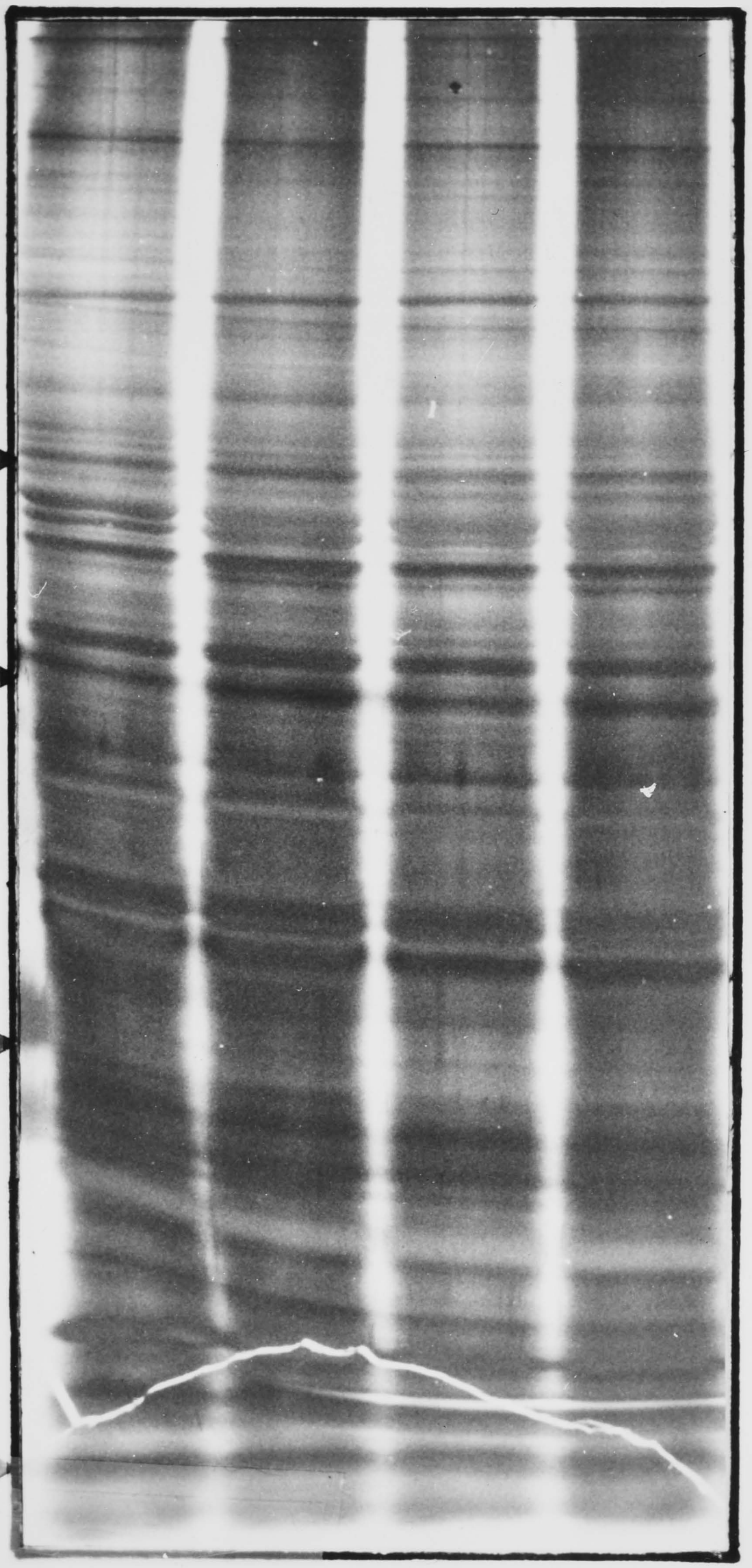
The enzymes assayed in this study are amongst the most abundant proteins in soybean nodules (see Verma et al. 1983) and therefore would be expected to be detectable using PAGE. However, there were no significant differences between Bragg and nts382 nodules in the levels of these enzymes. Therefore, none of them is likely to correspond to the protein which was characteristic of nts382 nodules.

There was no effect of nitrate treatment on the two dimensional PAGE profile of the nodule cytoplasmic proteins of Bragg. This was consistent with the absence of any effect of nitrate treatment on the in vitro activities of the enzymes involved in ammonia assimilation and ureide biosynthesis in Bragg nodules (see Chapter 4).

In summary, nts382 was less susceptible than Bragg to the inhibitory effect of delayed nitrate treatment on N_2 fixation. Nitrogenase activity, per g nodule fresh weight, of nts382 was several-fold less than that of Bragg. This was probably due to the lower bacteroid protein content of nts382 nodules.

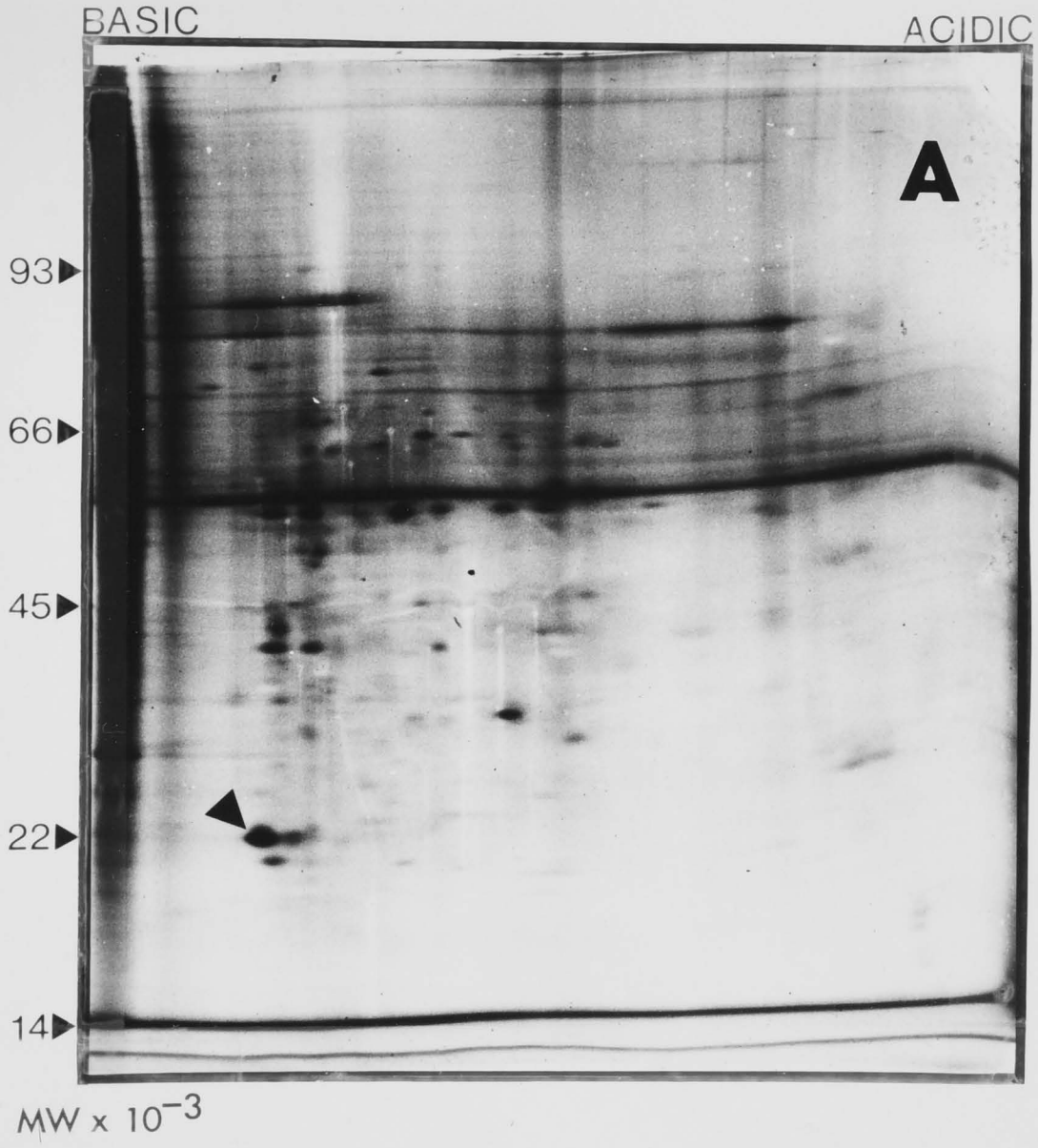
Although, whole plant nitrogenase activity of nts382 was no greater than that of Bragg, the ureide content of the xylem sap, leaves and nodules of the mutant was several-fold higher. The high ureide content of nts382 could not be attributed to elevated ureide biosynthesis in the roots or reduced ureide utilization in the leaves.

Fig. 6.1 Comparison of the Nodule Cytoplasmic Proteins of Bragg and nts382: One Dimensional SDS-PAGE. Plants were inoculated with Bradyrhizobium japonicum strain CB1809 and grown in 8 1 pots of sand/vermiculite (10 plants. pot^{-1}) in a naturally-illuminated glass-house during August-October, as described in Chapter 2. The pots were watered daily with water and three-times weekly with a nitrogen-free nutrient solution, until 67 DAP. The nutrient solution was then supplemented with either 10 mM KCl (control) or 10 mM KNO_3 (treated) and supplied for another 7 d, prior to harvest. Nodule extracts were prepared as described in section 2.10.1.2. Samples of the nodule extracts were prepared for one dimensional SDS-PAGE as described in section 2.10.1.3. Approximately 25 µg of protein was loaded per lane. Once run, the gels were silver stained (see section 2.10.3.2). Lane 1, nitrate treated Bragg plants; lane 2, control Bragg plants; lane 3, nitrate treated nts382 plants; lane 4, control nts382 plants.



MW x 10^{-3}

Fig. 6.2 Comparison of the Nodule Cytoplasmic Proteins of Bragg and nts382: Two Dimensional-PAGE. The nodule extracts were the same as those used to produce the gels shown in Fig. 6.1 but samples of the extracts were prepared for two dimensional PAGE as described in section 2.10.2.2. Approximately 100 µg of protein was loaded per first dimension IEF gel. The gels were silver stained (see section 2.10.3.2). (A) nts382, (B) The pH range of the IEF dimension was pH 3.5-10.


BASIC
ACIDIC
A
93
66
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MW x 10⁻³

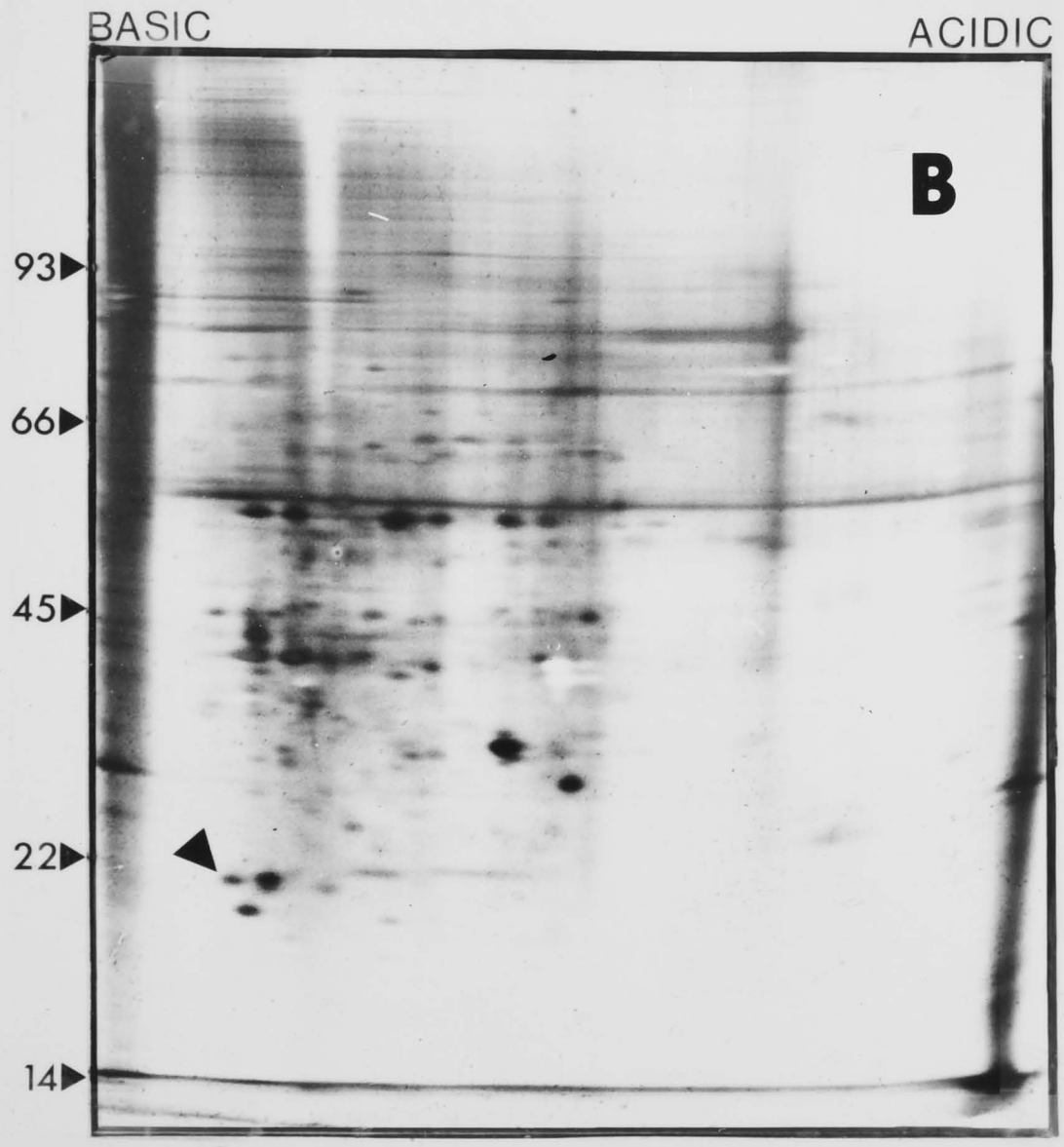
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Table 6.1 The Effect of Genotype and Delayed Nitrate Treatment on Nitrogenase Activity and Nodule Fresh Weight. The data are the average of seven separate experiments carried out between December and May. In some experiments, plants were inoculated with B. japonicum strain CB1809 and in others they were inoculated with strain USDA 110. All plants were grown in the absence of combined nitrogen in 8 l pots of either sand or a 1:1 mixture of sand:vermiculite (4-10 plants per pot) as described in Chapter 2. Nitrate treatment was commenced 6-8 weeks after planting with control plants receiving 10 mM KCl and treated 10 mM KNO_3, for two consecutive days prior to harvest. Nitrogenase activity was assayed using the standard acetylene reduction assay (see Chapter 2). Data are the mean ± SD (n=7).

Parameter	Bragg	nts382
Nitrogenase activity (μmol C_2H_4. h^{-1}. g^{-1} nodule FW)		
control	14.7±5.6	4.6±2.1
treated	3.6±1.5	2.0±0.9
% inhibition	75%±8%	51%±22%
Nitrogenase activity (μmol C_2H_4. h^{-1}. $plant^{-1}$)		
control	8.2±4.9	5.2±2.5
treated	1.8±0.4	2.4±1.4
% inhibition	73%±12%	48%±35%
Nodule fresh weight (g. $plant^{-1}$)		
control	0.56±0.25	1.24±0.40
treated	0.54±0.21	1.21±0.28

Table 6.2 The Effect of Genotype and Delayed Nitrate Treatment on Ureide Content of Xylem Sap and Nodules. Experimental details were as in the legend to Table 6.1. Five of the seven experiments used to produce the data shown in Table 6.1 included analyses of the ureide content of xylem sap and nodules. The results presented here are the average of these five experiments. Xylem exudate was collected and ureides and nitrate were analysed as described in Chapter 2. Data are the mean ± SD (n=5).

Parameter	Bragg	nts382
Xylem sap ureide content (μmol. ml^{-1} exudate)		
control	3.94±0.90	7.87±1.79
treated	1.54±1.00	4.74±2.64
% inhibition	62%±23%	41%±26%
Nodule ureide content (μmol. g^{-1} nodule FW)		
control	4.82±1.53	17.46±11.22
treated	4.18±0.89	17.76±8.50
Xylem sap nitrate content (μmol. ml^{-1} exudate)		
control	*ND	ND
treated	11.4±3.2	9.3±3.1

*ND = Not Detectable

Table 6.3 Enzymes Involved in Carbon Metabolism in Bragg and nts382 Nodules. Plants inoculated with B. japonicum strain CB1809 were grown in the absence of combined nitrogen in pots of sand as described in Chapter 2. Nodules were harvested 46-48 DAP, in late February. The enzyme activities were assayed in vitro as described in Chapter 2. Data are the mean ± SD of four replicate nodule extracts.

Enzyme Activity	Bragg	nts382
Alkaline invertase (μmol glucose. h^{-1}. g^{-1} nodule FW)	126±24	84±12
Fructokinase (μmol fructose-6-P. h^{-1}. g^{-1} nodule FW)	36.6±3.6	32.1±3.6
PEP Carboxylase (μmol PEP. h^{-1}. g^{-1} nodule FW)	378±42	300±12

Table 6.4 Leghaemoglobin and Bacteroid Protein Content of Bragg and nts382 Nodules. Plants inoculated with B. japonicum strain CB1809 were grown in the absence of combined nitrogen in pots of either vermiculite or a 1:1 mixture of sand:vermiculite as described in Chapter 2. Nodules were harvested 40-60 DAP and analysed for leghaemoglobin and bacteroid protein content (see Chapter 2). Data are the mean (± SD, n=4) of four separate experiments and were provided by D.A. Day.

Genotype	Leghaemoglobin (nmol haem. g^{-1} nodule FW)	Bacteroid Protein (mg bacteroid protein $.g^{-1}$ nodule FW)
Bragg	66.6± 9.8	34.3±7.4
nts382	31.8±11.8	14.9±7.5

Table 6.5 Enzymes of Purine Oxidation in the Nodules of Bragg and nts382. Plants inoculated with B. japonicum strain CB1809 were grown in the absence of combined nitrogen in pots of a 1:1 mixture of sand:vermiculite, as described in Chapter 2. Nodules were harvested 55-57 DAP, in mid-January. The enzyme activities were assayed in vitro as described in Chapter 2. Data are the mean ± SD of four replicate nodule extracts.

Enzyme Activity	Bragg	nts382
XDH (μmol NADH. h^{-1}. g^{-1} nodule FW)	1.92±0.30	1.62±0.18
Uricase (μmol uric acid. h^{-1}. g^{-1} nodule FW)	89.9±8.6	67.4±13.9
Allantoinase (μmol allantoic acid. h^{-1}. g^{-1} nodule FW)	67.7±9.4	55.8±4.3

Table 6.6 Ureide Content of the Leaves and Xylem Sap of Bragg and nts382. Plants were inoculated with B. japonicum strain CB1809 and grown in the absence of combined nitrogen in pots of a 1:1 mixture of sand:vermiculite as described in Chapter 2. Xylem exudate was collected from four plants in one pot per genotype 47 DAP, in December. The youngest fully expanded trifoliolate was taken from four plants in one pot per genotype 80 DAP, in January. The xylem exudate and the leaves were analysed for ureides as described in Chapter 2. Data are the mean ± SD (n=4).

Parameter	Bragg	nts382
Leaf ureides (μmol. g^{-1} leaf FW)		
-ureides	7.94±1.53	23.19±2.08
-allantoate	6.62±1.17	16.81±0.99
Xylem sap ureides (μmol. ml^{-1} exudate)		
-ureides	3.14±0.69	7.85±1.21
-allantoate	2.11±0.80	6.69±1.56

Table 6.7 Allantoin and Allantoate Degradation in the Leaves of Bragg and nts382. Plants inoculated with B. japonicum strain USDA 110 were grown in the absence of combined nitrogen in pots of a 1:1 mixture of sand:vermiculite as described in Chapter 2. Leaves were harvested 42-56 DAP, in March. The enzymes were assayed in vivo as described in Chapter 2. Data are the mean ± SD of two separate experiments.

Enzyme Activity	Bragg	nts382
Allantoinase (μmol allantoate. h^{-1}. g^{-1} leaf DW)	56±4	72±11
Allantoate hydrolysis (μmol glyoxylate. h^{-1}. g^{-1} leaf DW)	0.43±0.08	0.56±0.17

CHAPTER 7

The Effect of Inoculum Dose and Nitrate Treatment on Nodulation and N_2 Fixation of Soybean cv. Bragg and nts382

7.1 Introduction

The nitrate-tolerant soybean mutant nts382 is also supernodulating (Carroll et al. 1985 a, b, see also Chapters 5 and 6). The involvement, if any, of the supernodulation trait in the nitrate-tolerance of nts382 is not known. Interestingly, however, nodulation of nts382 can be reduced to the level of the parent cultivar Bragg by lowering the Bradyrhizobium inoculum dose (Day, Price, Schuller and Gresshoff, in preparation). This facilitates an assessment of the nitrate-tolerance of nts382 in the absence of supernodulation. This chapter, therefore, examines the interactive effect of inoculum dose and nitrate treatment on nodulation and nitrogenase activity of nts382 and its parent cultivar Bragg. In order to do this, Bragg and nts382 were inoculated with either a high or a low dose of Bradyrhizobium and two different nitrate treatments were then applied. The first (continuous nitrate treatment) involved supplying nitrate from the time of planting with the aim of determining the effect of nitrate treatment on nodule initiation and growth and the development of nitrogenase activity. The second (delayed nitrate treatment) involved supplying nitrate, for a short period of time, to an already established symbiosis in order to ascertain the effect of nitrate treatment on nitrogenase activity, independently of its effects on plant and nodule development.

7.2 Experimental

Seeds of soybean cv. Bragg and its mutant derivative nts382 were planted in 8 l pots of a 1:1 mixture of sand/vermiculite (4 seeds. pot^{-1}) in a naturally-illuminated glasshouse. The pots were inoculated with B. japonicum strain CB1809 at either 10^9 cells. pot^{-1} (high inoculum) or 10^3 cells. pot^{-1} (low inoculum). The plants receiving the continuous nitrate treatment were supplied daily, from the time of planting, with a nitrogen-free nutrient solution (see Chapter 2) supplemented with either 0.5 mM KNO_3 (low N) or 7.5 mM KNO_3 (high N). The high N plants were harvested 32 DAP and the low N plants 38 DAP, in late March. The plants receiving the delayed nitrate treatment were grown under the same conditions as the low N plants (see above) until 36 DAP, when they were treated with the nutrient solution supplemented with 10 mM KNO_3 (delayed N). The delayed nitrate treatment was continued for two consecutive days prior to harvest.

7.3 Results

7.3.1 Plant Growth

Preliminary experiments showed that dry matter content of plants dependent solely on N_2 as a nitrogen source was less than that of plants supplied with nitrate. This caused problems when comparing N_2-dependent and nitrate treated plants. The reduced growth of N_2-dependent plants was probably attributable to a transient period of nitrogen starvation prior to the onset of N_2 fixation and could be overcome by supplying nitrate at a level sufficient to relieve the nitrogen deficiency but not so high as to inhibit nodulation or N_2 fixation (see Carroll 1985). In

the experiments reported here the low N plants were dependent predominantly on N_2 as a nitrogen source but the low concentration of nitrate they received allowed them to overcome the transient period of nitrogen starvation prior to the onset of N_2 fixation. Table 7.1 displays the interactive effect of inoculum dose and continuous nitrate treatment on plant dry weight of Bragg and nts382. There was no significant difference in either shoot or root dry weight between the low N and high N plants. Neither was there any significant effect of inoculum dose on either shoot or root dry weight. There were, however, differences in dry matter content between the genotypes. Shoot and root (excluding nodules) dry weight were approximately two-fold greater for Bragg than they were for nts382.

7.3.2 Nodulation

The interactive effect of inoculum dose and continuous nitrate treatment on nodulation of Bragg and nts382 is illustrated in Table 7.2. Under high inoculum conditions, nts382 had almost six-fold (low N) and eight-fold (high N) more nodules than Bragg. Under low inoculum conditions, however, nts382 had a more or less similar number of nodules to Bragg. High N reduced nodule number of Bragg by 52% (high inoculum) and 46% (low inoculum). Nodule number of nts382 was reduced by 36% (high inoculum) and 24% (low inoculum). Thus, nodule initiation of nts382 was less markedly affected by nitrate treatment than was nodule initiation of Bragg.

Under high inoculum conditions, nodule fresh weight of low N nts382 plants was approximately two-fold greater than that of low N Bragg plants. Under low inoculum, low N conditions, on the other hand, nodule

fresh weight of nts382 was similar to that of Bragg under high inoculum, low N conditions. High N enhanced the difference between the genotypes with nodule fresh weight of high and low inoculum nts382 being six- and two-fold, respectively, greater than that of high and low inoculum Bragg.

This enhancement of the difference between the genotypes was a reflection of the differential effect of high N on nodule growth of Bragg and nts382. Nodule fresh weight of both high and low inoculum nts382 was not significantly affected by high N whereas that of Bragg was reduced by 76% (high inoculum) and 59% (low inoculum). From the data presented it is apparent that the nitrate tolerance of nodulation of nts382 is independent of the inoculum dose. In other words, when nodulation was reduced due to a reduction in the inoculum dose, nts382 retained its ability to nodulate in the presence of high levels of nitrate. In contrast, nodulation of Bragg was markedly inhibited by the high nitrate levels under both high and low inoculum conditions.

In the case of the low N, high inoculum plants, nodule number of nts382 was six-fold greater than that of Bragg whereas nodule fresh weight was only two-fold greater. This shows that nts382 had smaller as well as many more nodules than Bragg. The specific mass of nts382 and Bragg nodules, under high inoculum conditions, averaged 3.1 mg and 10.0 mg, respectively. However, when nodule number of nts382 was reduced in response to a reduction in inoculum dose there was a corresponding increase in the individual mass of the nodules of the mutant to an average value of 9.8 mg. Thus, the individual nodule mass of low inoculum nts382 resembled that of high inoculum Bragg. Clearly, nodule size was inversely proportional to nodule number.

7.3.3 Nitrogenase Activity

Table 7.3 illustrates the interactive effect of inoculum dose and continuous nitrate treatment on nitrogenase activity per g nodule fresh weight. For low N plants, activity of high inoculum Bragg was approximately seven-fold greater than that of high inoculum nts382. Lowering the inoculum dose resulted in an almost three-fold increase in activity of nts382. In contrast, there was no significant effect of inoculum dose on activity of Bragg.

Nitrogenase activity, per g nodule fresh weight, of Bragg was not significantly affected by high N. In contrast, activity of nts382 was markedly stimulated by high N. High N in combination with reduced inoculum, increased activity of nts382 to the level of Bragg. Under high inoculum conditions, high N stimulated activity of nts382 three-fold. Under low inoculum conditions, the increase was only two-fold. Thus, activity of low inoculum nts382 was less markedly stimulated than that of high inoculum nts382. The nitrate-response of low inoculum nts382 was intermediate between Bragg and high inoculum nts382.

In order to investigate the effect of nitrate treatment on nitrogenase activity, independently of its effect on nodulation, a high concentration of nitrate (10 mM) was supplied to some of the low N plants for 2 d prior to harvest (delayed nitrate treatment). This treatment did not significantly affect plant dry weight or nodule fresh weight (Table 7.4). Under the high inoculum regime, delayed nitrate treatment inhibited nitrogenase activity, per g nodule fresh weight, of Bragg and nts382 by 87% and 43%, respectively (Table 7.5). Activity of control nts382 plants was very low, however, and when it was increased

in response to a reduction in the inoculum dose, nitrate induced inhibition increased to 74%. No such interaction between inoculum dose and nitrate-inhibition was seen in Bragg. Thus, nitrogenase activity of low inoculum nts382 was almost as susceptible to nitrate inhibition as was that of high inoculum Bragg.

7.3.4 Estimated Bacteroid Protein Content of Nodules

The low nitrogenase activity, per g nodule fresh weight, of high inoculum nts382 could be almost entirely accounted for by the low bacteroid protein content of the nodules (Table 7.6, see also Chapter 6). Furthermore, the increase in nitrogenase activity of nts382, resulting from a reduction in the inoculum dose, was associated with an increase in the bacteroid protein content of the nodules (Table 7.6).

7.3.5 Xylem Sap Ureide Content

Table 7.7 shows the interactive effect of inoculum dose and continuous nitrate treatment on nitrogenase activity per plant and the concentration of ureides in the xylem sap. Under both high and low inoculum conditions, whole plant nitrogenase activity of low N Bragg plants was more than four-fold greater than that of low N nts382 plants. Despite this, however, xylem sap ureide concentration of Bragg was only 59% of that of nts382. Reducing the inoculum dose had no significant effect on either nitrogenase activity per plant or xylem sap ureide concentration.

In section 7.3.1 it was noted that the shoot dry matter content of nts382 was less than half of that of Bragg. In view of the above

results, this can probably be attributed to the lower whole plant nitrogenase activity of nts382.

The low whole plant nitrogenase of nts382, found in the present study, is in contrast to the results presented in Chapter 6. It is also in contrast to the results of previous studies in which whole plant nitrogenase activity was similar for Bragg and nts382 (Carroll et al. 1985 a, b, Day et al. 1986). One explanation for the discrepancy between the studies may lie in the time of year in which they were carried out. In other words, seasonal factors, most probably light intensity, may have a profound effect on nitrogenase activity of nts382.

Continuous nitrate treatment inhibited whole plant nitrogenase activity of Bragg by 60%-80% whereas it stimulated that of nts382 two- to three-fold. The effect of continuous nitrate treatment on xylem sap ureide concentration of Bragg was proportionally similar to the effect on whole plant nitrogenase activity. In contrast, there was no significant effect of continuous nitrate treatment on xylem sap ureide concentration of nts382 despite the two- to three-fold stimulation of whole plant nitrogenase activity of the mutant.

The interactive effect of inoculum dose and delayed nitrate treatment on nitrogenase activity per plant and xylem sap ureide concentration is shown in Table 7.8. Delayed nitrate treatment inhibited whole plant nitrogenase activity and reduced xylem sap ureide concentration of both Bragg and nts382. This contrasted with the results obtained with continuous nitrate treatment. However, the effect on nts382 was not as great as the effect on Bragg. Whole plant nitrogenase activity of Bragg was inhibited by 89% (high inoculum) and 84% (low inoculum) and that of nts382 was inhibited by 62% (high

inoculum) and 68% (low inoculum). The reductions in xylem sap ureide concentration were similar although slightly less marked. There was no effect of inoculum level on the magnitude of the inhibitory effect of delayed nitrate treatment on nitrogenase activity per plant. This was consistent with the results obtained with continuous nitrate treatment, but it was in contrast to the results obtained when nitrogenase activity was expressed on a nodule fresh weight basis.

7.3.6 Nodule Ureide Content

Table 7.9 illustrates the interactive effect of inoculum dose and continuous nitrate treatment on nodule ureide content. Under high inoculum conditions, nodule ureide content of control nts382 plants was more than five-fold greater than that of control Bragg plants whereas nitrogenase activity, expressed on the same basis (per g nodule fresh weight), was more than seven-fold less. In contrast, under low inoculum conditions, nodule ureide content of nts382 was only two-fold greater and nitrogenase activity was only three-fold less. Continuous nitrate treatment lowered the ureide content of low inoculum Bragg and nts382 nodules by 83% and 76%, respectively. The reduction in nodule ureide content of high inoculum nts382 was not so marked, however, being only 43%. When the inoculum dose was reduced, nodule ureide content of control Bragg plants was increased whereas that of control nts382 plants was decreased. Concomitant with the reduction in the ureide content of nts382 nodules was an increase in nitrogenase activity and bacteroid protein content of the nodules, expressed on a nodule fresh weight basis (Table 7.6). A similar reduction in nodule ureide content was seen when

nitrogenase activity, per g nodule fresh weight, was increased in response to continuous nitrate treatment.

7.3.7 Xylem Sap Nitrate Content and *In Vivo* Leaf Nitrate Reductase Activity

Xylem sap nitrate concentration and *in vivo* leaf nitrate reductase activity (NRA) of plants receiving the continuous nitrate treatment are shown in Table 7.10. The concentration of nitrate in the xylem exudate of Bragg was two-fold less than it was in the xylem exudate of *nts*382. Similarly, NRA in the leaves (assayed *in vivo* in the absence of added nitrate) was somewhat less for Bragg than it was for *nts*382. These results indicate that the nitrate tolerance of nodulation of *nts*382 cannot be explained either by decreased nitrate uptake (as indicated by the xylem sap nitrate concentration) or by a lower level of NRA in the leaves. Both xylem sap nitrate concentration and NRA were less for low inoculum Bragg, as compared with high inoculum Bragg. There is no obvious explanation for this, except that it may be due to differences in xylem sap exudation rate.

7.4 Discussion

Under high inoculum conditions, *nts*382 was supernodulated and its nitrogenase activity, expressed on a nodule fresh weight basis, was several-fold lower than that of the parent cultivar Bragg. The low nitrogenase activity, per g nodule fresh weight, of *nts*382 could be almost entirely accounted for by the low bacteroid protein content of its nodules. Decreasing the inoculum dose reduced nodule number and nodule fresh weight, per plant, of *nts*382 to the level of Bragg. The

reduction in nodule number was accompanied by an increase in individual nodule mass, nitrogenase activity, expressed on a nodule fresh weight basis, and bacteroid protein content of the nodules.

The previously reported nitrate tolerance of nodulation of nts382 (Carroll et al. 1985 a, b) was confirmed. In addition, when compared with Bragg, nitrogenase activity of nts382 was shown to be less susceptible to inhibition by delayed nitrate treatment (see also Chapters 5 and 6). However, the degree of nitrate tolerance of nitrogenase activity of nts382 was dependent on the Rhizobium inoculum level. Lowering the inoculum level resulted in an increase in nitrogenase activity, per g nodule fresh weight, of low N nts382 plants, along with an increase in the susceptibility of nitrogenase activity to inhibition by delayed nitrate treatment. This phenomenon was not observed for Bragg. Therefore the reduced susceptibility of nitrogenase activity of nts382 to inhibition by delayed nitrate treatment appears, at least in part, to be related to the supernodulation phenotype.

In contrast, the nitrate tolerance of nodulation of nts382 was apparently unaffected by the inoculum dose. These observations suggest that the reduced nitrate susceptibility of nitrogenase activity of high inoculum nts382 may be related to the altered morphology of nts382 nodules under high inoculum conditions, rather than any other factor.

The reduced susceptibility of nts382 to the inhibitory effects of nitrate on nodulation (high and low inoculum conditions) and nitrogenase activity (high inoculum conditions only) could not be explained either by decreased nitrate uptake (as indicated by the nitrate content of xylem exudate) or by decreased NRA in the leaves (see also Chapter 6). The lower level of nitrate in the xylem exudate of Bragg, as compared

with nts382, may indicate that the same amount of nitrate was being taken up by the two genotypes but more was being reduced in the roots of Bragg, than in the roots of nts382. If this is so, then the α-amino-nitrogen content of the xylem sap of Bragg would be expected to be higher than that of nts382. Unfortunately, insufficient xylem sap was available to determine this. Another way of assessing nitrate uptake would be to measure nitrate depletion from the nutrient solution in a hydroponic plant culture system.

Supernodulation of nts382 has been attributed to a mutation in the gene(s) controlling autoregulation of nodulation (Carroll et al. 1985 a, Gresshoff and Delves 1986). Delves et al. (1986) showed that super-nodulation is controlled by a signal which originates in the shoot. Recent evidence suggests that rather than producing a positive regulatory signal which stimulates nodulation, nts382 is characterised by its failure to produce a negative regulatory signal which suppresses nodulation (J.E. Olsson, pers. comm.). It is conceivable that besides permitting supernodulation, the failure of nts382 to produce this signal may also permit nodulation and N_2 fixation in the presence of nitrate.

On the basis of altered growth characteristics of the mutant, including increased lateral root formation and reduced shoot growth, in either the presence or absence of Rhizobium, it has been proposed that the shoot signal is itself a phytohormone or else it affects phytohormone biosynthesis or action (Day et al. 1986, Gresshoff and Delves 1986). The major growth promoting phytohormones synthesised in plant shoots are auxins and gibberelins (see Moore 1979). The other growth promoting phytohormones, the cytokinins, are synthesised predominantly in the meristematic regions of the roots. The increased

lateral root formation of nts382 is consistent with a high level of auxins and/or a low level of cytokinins. Furthermore, both auxins and cytokinins have been implicated in cortical cell proliferation in response to inoculation with Rhizobium (see Libbenga and Bogers 1974, see also Newcomb 1980) and exogenously applied IAA (an auxin) overcomes the inhibitory effect of nitrate on nodulation (Valera and Alexander 1965, Munns 1968). Therefore, both supernodulation and nitrate tolerance of nodulation of nts382 may be the result of an alteration in phytohormone balance in the mutant. Although, under high inoculum conditions, nitrogenase activity, per g nodule fresh weight, of nts382 was less than that of Bragg, xylem sap and nodule ureide content were greater. This confirms the observations made in Chapter 6. Decreasing the inoculum dose resulted in a reduction in xylem sap and nodule ureide content of nts382. This was concomitant with the increase in nitrogenase activity, per g nodule fresh weight, of the mutant and reversion of the nodulation phenotype of nts382 to that of Bragg. Therefore, high levels of ureides in the mutant appear to be associated with supernodulation.

Under the experimental conditions employed here, there was no inhibitory effect of continuous nitrate treatment on nitrogenase activity, per g nodule fresh weight, of either Bragg or nts382. It would be interesting, therefore, to determine whether there is a nitrate concentration which inhibits the development of nitrogenase activity of Bragg but not nts382. It would then be possible to ascertain whether development of nitrogenase activity in nts382 is nitrate tolerant like nodule development.

In conclusion, lowering the inoculum dose resulted in a reduction in nodulation of nts382. This was coupled with an increase in nitrogenase activity, per g nodule fresh weight, and nodule bacteroid content and a decrease in nodule ureide content. These effects were not seen with Bragg. Nodulation of nts382 was nitrate-tolerant under both high and low inoculum conditions. Nitrogenase activity, on the other hand, only showed reduced susceptibility to the inhibitory effect of delayed nitrate treatment under high inoculum conditions. Supernodulation and nitrate-tolerance of nodulation are proposed to be due to an alteration in the ratio of auxins to cytokinins in the mutant. The reduced nitrate-susceptibility of nitrogenase activity of high inoculum nts382 may be due to an alteration in nodule morphology.

Table 7.1 The Interactive Effect of Inoculum Dose and Continuous Nitrate Treatment on Shoot and Root (Excluding Nodules) Dry Weight.

Plants received the continuous nitrate treatment as described in section 7.2. Either two (low N) or four (high N) plants from each of two pots per treatment were harvested, separated into roots and shoots and dried at 80°C for two days. Data are the mean ± SD (n=4).

Genotype-Inoculum	Shoot Dry Weight (g. plant^{-1})		Root Dry Weight (excluding nodules) (g. plant^{-1})	
	Low N	High N	Low N	High N
Bragg-high	2.36±0.28	2.26±0.67	0.43±0.16	0.42±0.03
Bragg-low	2.14±0.49	2.46±0.43	0.49±0.18	0.83±0.31
nts382-high	0.98±0.27	1.16±0.12	0.17±0.05	0.21±0.03
nts382-low	1.02±0.32	1.49±0.39	0.21±0.07	0.27±0.13

Table 7.2 The Interactive Effect of Inoculum Dose and Continuous Nitrate Treatment on Nodulation. Plants received the continuous nitrate treatment as described in section 7.2. Either two (low N) or four (high N) plants were harvested from each of two pots per treatment and the nodules were detached, counted and weighed. Data are the mean ± SD (n=4).

Genotype-Inoculum	Nodule Number plant^{-1}		Nodule Fresh Weight (g. plant^{-1})	
	Low N	High N	Low N	High N
Bragg-high	107±17	51±21	1.07±0.17	0.26±0.05
Bragg-low	46±19	25±11	0.93±0.29	0.38±0.15
nts382-high	608±302	389±118	1.91±0.55	1.62±0.33
nts382-low	99±8	75±18	0.97±0.22	0.84±0.14

Table 7.3 The Interactive Effect of Inoculum Dose and Continuous Nitrate Treatment on Nitrogenase Activity. Plants received the continuous nitrate treatment as described in section 7.2. The nodulated roots of either two (low N) or four (high N) plants from each of two pots per treatment were assayed for nitrogenase activity using the standard acetylene reduction assay (see Chapter 2). Data are the mean ± SD (n=4).

Genotype-Inoculum	Nitrogenase Activity (μmol $C_2H_4 . h^{-1} . g^{-1}$ nodule fresh weight)	
	Low N	High N
Bragg-high	15.5±6.0	16.1±4.1
Bragg-low	18.2±5.0	15.3±0.7
nts382-high	2.1±0.4	6.8±1.8
nts382-low	5.8±1.2	13.0±1.2

Table 7.4 The Interactive Effect of Inoculum Dose and Delayed Nitrate Treatment on Plant Dry Weight and Nodule Fresh Weight. Plants received the delayed nitrate treatment as described in section 7.2. Two plants were harvested from each of two pots per treatment. Data are the mean ± SD (n=4).

	Plant Dry Weight (minus nodules) ($g.\ plant^{-1}$)		Nodule Fresh Weight ($g.\ plant^{-1}$)	
Genotype-Inoculum	Low N	Delayed N	Low N	Delayed N
Bragg-high	1.40±0.17	1.54±0.29	1.07±0.17	0.87±0.19
Bragg-low	1.31±0.32	1.66±0.29	0.93±0.29	1.14±0.19
*nts*382-high	0.59±0.16	0.56±0.09	1.91±0.55	1.31±0.29
*nts*382-low	0.59±0.17	0.83±0.09	0.97±0.22	1.27±0.13

Table 7.5 The Interactive Effect of Inoculum Dose and Delayed Nitrate Treatment on Nitrogenase Activity. Plants received the delayed nitrate treatment as described in section 7.2. The nodulated roots of two plants from each of two pots per treatment were assayed for nitrogenase activity using the standard acetylene reduction assay (see Chapter 2). Data are the mean ± SD (n=4).

Genotype-Inoculum	Nitrogenase Activity (μmol C_2H_4. h^{-1}. g^{-1} nodule fresh weight)	
	Low N	Delayed N
Bragg-high	15.5±6.0	2.0±0.4
Bragg-low	18.2±5.0	2.7±0.5
nts382-high	2.1±0.4	1.2±0.2
nts382-low	5.8±1.2	1.5±0.9

Table 7.6 The Interactive Effect of Inoculum Dose and Continuous Nitrate Treatment on Bacteroid Protein Content of Nodules and Nitrogenase Activity per mg Bacteroid Protein. The plants were the same as those used to produce the data shown in Table 7.3. Data are the mean ± SD (n=4). The bacteroid protein content of the nodules was determined by D.A. Day using β-HBDH as a bacteroid specific marker (see Chapter 2). The nitrogenase data used to calculate nitrogenase activity on a mg bacteroid protein basis are taken from Table 7.3.

	Bacteroid Protein (mg. g^{-1} nodule FW)		Nitrogenase Activity (nmol C_2H_4. h^{-1}. mg^{-1} bact. protein)	
Genotype-Inoculum	Low N	High N	Low N	High N
Bragg-high	30.4	29.7	511±171	541±135
Bragg-low	40.6	37.0	448±106	413±15
nts382-high	5.6	14.0	371±61	482±84
nts382-low	20.4	26.6	308±55	490±51

Table 7.7 The Interactive Effect of Inoculum Dose and Continuous Nitrate Treatment on Nitrogenase Activity and Xylem Sap Ureide Content. Plants received the continuous nitrate treatment as described in section 7.2. The nitrogenase data are the same as those presented in Table 7.3, except that they are expressed on a per plant basis instead of a nodule fresh weight basis. Xylem exudate was collected from a total of 2-6 plants from either one (low N) or two (high N) pots and analysed for ureides as described in Chapter 2. Data are the mean ± SD.

	Nitrogenase Activity (μmol C_2H_4. h^{-1}. $plant^{-1}$)		Xylem Sap Ureides (μmol. ml^{-1} xylem sap)	
Genotype-Inoculum	Low N	High N	Low N	High N
Bragg-high	16.9±7.8	4.2±1.4	2.9±0.6	0.6±0.1
Bragg-low	18.0±8.9	5.8±2.2	2.6±0.2	0.9±0.3
nts382-high	3.9±0.9	10.7±3.1	4.9±0.6	4.7±0.4
nts382-low	5.7±2.3	10.9±1.5	3.9±1.7	4.4±1.0

Table 7.8 The Interactive Effect of Inoculum Dose and Delayed Nitrate Treatment on Nitrogenase Activity and Xylem Sap Ureide Content. Plants received the delayed nitrate treatment as described in section 7.2. The nitrogenase data are the same as those presented in Table 7.5, except, that they are expressed on a whole plant basis instead of a nodule fresh weight basis. Xylem exudate was collected from a total of 3-4 plants from one pot and analysed for ureides as described in Chapter 2. Data are the mean ± SD.

	Nitrogenase Activity (μmol $C_2H_4 . h^{-1} . plant^{-1}$)		Xylem Sap Ureide Content (μmol. ml^{-1} xylem sap)	
Genotype-Inoculum	Low N	Delayed N	Low N	Delayed N
Bragg-high	16.9±7.8	1.8±0.6	2.9±0.6	0.7±0.2
Bragg-low	18.0±8.9	2.8±0.3	2.6±0.2	0.6±0.2
nts382-high	3.9±0.9	1.5±0.1	4.9±0.6	2.6±0.6
nts382-low	5.7±2.3	1.8±0.9	3.9±1.7	1.2±0.5

Table 7.9 The Interactive Effect of Inoculum Dose and Continuous Nitrate Treatment on Nitrogenase Activity and Nodule Ureide Content.

Plants received the continuous nitrate treatment as described in section 7.2. The nitrogenase data are the same as those shown in Table 7.3. Nodules detached from the root systems assayed for nitrogenase activity were analysed for ureides as described in Chapter 2. Data are the mean ± SD (n=4).

	Nitrogenase Activity (μmol C_2H_4. h^{-1}. g^{-1} nodule FW)		Nodule Ureide Content (μmol. g^{-1} nodule FW)	
Genotype-Inoculum	Low N	High N	Low N	High N
Bragg-high	15.5±6.0	16.1±4.1	6.5±0.7	*NA
Bragg-low	18.2±5.0	15.3±0.7	10.9±1.3	1.9±0.3
nts382-high	2.1±0.4	6.8±1.8	35.5±1.7	20.4±0.6
nts382-low	5.8±1.2	13.0±1.2	21.0±1.5	5.1±0.5

*NA = Not Analysed.

Table 7.10 The Effect of Continuous Nitrate Treatment on Xylem Sap Nitrate Concentration and *In Vivo* Leaf NRA. Plants were grown as described in section 7.2 with 7.5 mM nitrate being supplied daily from the time of planting. Xylem exudate was collected from 3-8 plants (see figures in parentheses) from two pots per treatment and analysed for nitrate as described in Chapter 2. Leaf NRA was assayed, *in vivo*, in the absence of exogenous nitrate, as described in Chapter 2. Leaf disks were collected from four plants in each of four pots per treatment and each pot therefore constituted one replicate. Data are the mean ± SD.

Genotype-Inoculum	Xylem Nitrate (μmol. ml^{-1} exudate)	*In vivo* Leaf NRA (μmol NO_2^-. h^{-1}. g^{-1} leaf DW)
Bragg-high	4.67±1.08(6)	2.36±1.14
Bragg-low	2.98±0.22(8)	0.16±0.21
*nts*382-high	8.70±0.90(3)	8.69±3.18
*nts*382-low	9.19±1.40(4)	6.33±2.07

CHAPTER 8

General Discussion

After many years of investigation the event(s) responsible for the inhibitory effect(s) of nitrate on nodulation and N_2 fixation is (are) still not known. This thesis focused predominantly on the inhibitory effect of nitrate on N_2 fixation rather than nodulation. However, it did show that the mechanism involved in nitrate-inhibition of nodulation is probably different from that which is involved in the inhibitory effect of nitrate on N_2 fixation. Firstly, soybean cultivars which showed improved ability to nodulate in the presence of high levels of nitrate did not all show reduced susceptibility to the inhibitory effect of delayed nitrate treatment on N_2 fixation. Secondly, nodulation of the supernodulating soybean mutant nts382 was not inhibited by nitrate (continuous treatment) whereas N_2 fixation was (delayed nitrate treatment).

Nitrate inhibition of nodulation is a multi-faceted phenomenon. It involves inhibitory effects on Rhizobium bacterial infection of legume roots as well as on nodule development (see Pate and Atkins 1983). The inhibitory effect of nitrate on root infection has been attributed to a direct effect of nitrate itself, since the inhibition is the same whether plants are inoculated with nitrate reductase expressing or nitrate reductase deficient Rhizobium strains (Gibson and Pagan 1977). Nitrate has been postulated to have its effect on infection by inhibiting the accumulation of lectins in root cell walls (see Dazzo and Gardiol 1984). Lectins are thought to be involved in the infection of

legume roots by Rhizobium bacteria. Changes in the chemical composition of the root cell walls themselves have also been seen in response to nitrate treatment (see Dazzo and Gardiol 1984). This suggests that nitrate may have a phytohormonal effect.

The inhibitory effect of nitrate on N_2 fixation, on the other hand, has been postulated to involve either "photosynthate deprivation" (Oghoghorie and Pate 1971) or "nitrite poisoning" (Rigaud and Puppo 1977, Trinchant and Rigaud 1980). Contrary to the photosynthate deprivation hypothesis, the recent observations of Wasfi and Prioul (1986) suggest that any decline in photosynthate translocation to the nodules is a result rather than the cause of the nitrate induced decline in nitrogenase activity, in soybean. Similarly, the present study found no correlation between nitrate reductase activity in the leaves and the level of nitrate-induced inhibition of nitrogenase activity. Neither was there any nitrate-induced decline in the in vitro activities of alkaline invertase, fructokinase and PEP carboxylase; some of the enzymes in the plant fraction of the nodule which are involved in the provision of respiratory substrates to the bacteroids.

Nitrite produced by the bacteroid nitrate reductase is thought not to be involved in the inhibitory effect of nitrate on nitrogenase activity (Gibson and Pagan 1977, Stephens and Neyra 1983, Streeter 1985 a, b). However, nitrite produced in the nodule cytoplasm may still be involved. If this is so then it suggests compartmentation of nitrite in the nodule and an interaction between nitrite and leghaemoglobin (Rigaud and Puppo 1977) rather than between nitrite and nitrogenase (Trinchant and Rigaud 1980).

If the "nitrite poisoning" hypothesis is to be validated then nitrite needs to be shown to bind to leghaemoglobin *in situ*. This could be done by performing spectral analyses of leghaemoglobin in intact nodules. Klucas *et al*. (1985) have succeeded in obtaining spectra of leghaemoglobin in small intact nodules by placing the whole plant in the modified sample compartment of a spectrophotometer. However, they report little success with bigger nodules, such as those of soybean. Nevertheless, they suggest that, if soybean nodules were grown between two glass plates, for example, so that they were thinner in one dimension, it may be possible to obtain leghaemoglobin spectra for these nodules as well.

Another approach to the testing of the "nitrite poisoning" hypothesis would involve the use of nitrate reductase deficient *Rhizobium* mutants in association with plant mutants lacking nodule nitrate reductase activity. A soybean mutant, nr_1, lacking the constitutive nitrate reductase in the leaves, had a similar level of nitrogenase (acetylene reduction) activity to its wild type parent, when grown in the field (Ryan *et al*. 1983). Likewise, when grown in pots, in the presence of nitrate, nodulation and nodule fresh weight of this mutant were inhibited similarly to the wild-type parent (Carroll and Gresshoff 1986). Another soybean mutant, NR328, with reduced constitutive nitrate reductase activity in the leaves also showed decreased nodulation in the presence of nitrate (Carroll and Gresshoff 1986). The nr_1 mutant grew equally as well as the wild type on nitrate but growth of NR328 was reduced. The good growth of nr_1 indicates that the inducible nitrate reductase activity was sufficient to satisfy the nitrogen requirements of the plant. The reduced growth of NR328 was

probably due to the accumulation of nitrate in the tissues of this mutant (Whitmore-Smith 1985). Furthermore, the accumulation of nitrate suggests that this mutant may also be impaired in its inducible nitrate reductase (B.J. Carroll, pers. comm.). Clearly, it will be necessary to obtain mutants which lack both the constitutive and the inducible nitrate reductase before any conclusions can be drawn regarding the involvement of nitrate reduction and nitrite in the inhibitory effect of nitrate on nodulation and N_2 fixation. The nitrate reductase activity in soybean nodules is apparently constitutive (Hunter 1983) whereas only an inducible nitrate reductase activity is found in the roots of soybean (Nelson et al. 1983). Therefore, the root and nodule enzymes are probably different. Consequently, it will most likely require separate mutagenic events to produce soybeans lacking both root and nodule nitrate reductase activity.

As an alternative to the hypotheses discussed above, Carroll (1985) proposed that the nitrate-induced decline in nitrogenase activity may be due to the formation of a physical barrier to O_2 diffusion into the nodule, resulting in O_2 being limiting for bacteroid respiration. The evidence he cites for this is that increasing the O_2 tension in a closed assay system relieves the inhibitory effect of nitrate on nitrogenase (acetylene reduction) activity. An alternative interpretation of this observation is that nitrite binds to a proportion of the leghaemoglobin in the nodules thus decreasing O_2 flux to the bacteroids; increasing the O_2 tension would restore O_2 flux.

The same effect was seen with dark-treated soybean plants (Carroll 1985). However, whereas nitrate treatment does not affect leghaemoglobin levels (this study), dark treatment results in a rapid decline in

both nitrogenase activity and leghaemoglobin levels in soybean nodules (Pfeiffer et al. 1983). Therefore, the effects of increased O_2 tension are equally consistent with the involvement of either a physical barrier or a decline in the level of "functional" leghaemoglobin.

Yet another interpretation of the observations of Carroll (1985) is that both nitrate and dark treatment reduce photosynthate translocation to the nodules and increasing the O_2 tension stimulates mobilization of stored carbohydrate in the nodules. This interpretation is based on the observation that nitrogenase activity is dependent on currently supplied photosynthate (Lawrie and Wheeler 1975, Antoniw and Sprent 1978, Reibach and Streeter 1983, Gordon et al. 1985, Kouchi et al. 1985) under what are probably carbon sufficient, O_2 limited conditions. However, when carbon is limiting, mobilization of stored carbohydrates may be stimulated by increasing O_2 availability.

Replacement of N_2 with either acetylene or argon results in a decline in nitrogenase (acetylene reduction) activity and nodule respiration, in some legumes (Minchin et al. 1983). Since it could be reversed by increasing the O_2 tension, the decline in nitrogenase activity and nodule respiration was postulated to be due to the formation of a barrier to O_2 diffusion into the nodule (Witty et al. 1984). The effect was observed with white clover and some other legumes but probably does not occur in soybean cv. Bragg (this study). Neither did it appear to occur in white clover plants which had been pre-treated with nitrate (Witty et al. 1984). Based on the observations of Carroll (1985), this suggests that the barrier to O_2 diffusion may already be in place in nitrate inhibited nodules.

The reduced nitrate susceptibility of nitrogenase activity of nts382, under high inoculum conditions, may be due to altered nodule morphology. The morphology of nts382 nodules, under high inoculum conditions, is suggestive of nodules whose development has been arrested at a juvenile stage. Firstly, they are smaller than Bragg nodules. Secondly, their infected area and the number of bacteroids per infected cell is low (Day, Price, Schuller and Gresshoff, in preparation). Thirdly, their leghaemoglobin content is low. The small size of high inoculum nts382 nodules means that their surface area to volume ratio is high and their low bacteroid content probably means that the rate of respiration per nodule is low. Both of these factors would be expected to result in a higher O_2 concentration in the vicinity of the bacteroids and consequent inactivation of nitrogenase. However, nitrogenase is not inactivated since activity expressed on a bacteroid protein basis is the same as that of Bragg nodules. This suggests that there is a barrier to O_2 diffusion into nts382 nodules which prevents inactivation of nitrogenase. This barrier may either be the physical barrier which Carroll (1985) suggests or it may be a consequence of the low leghaemoglobin content of nts382 nodules. Either way, high inoculum nts382 nodules can be likened to nitrate inhibited Bragg nodules. This may explain why the inhibitory effect of nitrate on nitrogenase activity is less pronounced in high inoculum nts382 than it is in Bragg.

Bacteroids are dependent on their host plant for a carbon source, probably carboxylic acids, to support nitrogenase activity. The most important evidence favouring carboxylic acids as the form in which carbon is supplied to the bacteroids is as follows. Firstly, carboxylic acids stimulate nitrogenase activity of isolated bacteroids (Bergersen

and Turner 1967, Trinchant et al. 1981, McNeil et al. 1984). Secondly, bacteroids which are either impaired in carboxylic acid uptake or have a defective TCA cycle, are ineffective in fixing nitrogen (see Ronson and Astwood 1985). Thirdly, gluconeogenic enzymes are repressed in the bacteroid state and bacteroids lacking phosphoenopyruvate carboxykinase, a key enzyme in gluconeogenesis, are still capable of fixing nitrogen (McKay et al. 1985).

Therefore, another way in which nitrate may inhibit nitrogenase activity is by inhibiting carboxylic acid uptake. This could occur at the peribacteroid membrane. Proton-pumping ATPases have been found in both the plasma membrane and the tonoplast of soybean roots (Lew and Spanswick 1985). The plasma membrane-type ATPase was characterised by its sensitivity to vanadate inhibition whereas the tonoplast type ATPase was inhibited by nitrate. An ATPase found in the peribacteroid membrane of soybean nodules was inhibited by vanadate, did not cross-react with antinodulin antiserum, and was therefore postulated to be a plasma membrane-type ATPase (Blumwald et al. 1985). The effect of nitrate on this ATPase was not investigated. Acidification of the peribacteroid space, mediated by the peribacteroid membrane ATPase, was postulated to promote the conversion of NH_3 to NH_4^+ and thereby facilitate the removal of NH_3 from the bacteroids by creating an NH_3 diffusion gradient. Ammonium was seen as being transported into the host cytoplasm either directly via the ATPase or indirectly via an amine uniport (Blumwald et al. 1985).

Clearly, if the peribacteroid membrane ATPase acidifies the peribacteroid space it would be expected to promote the alkalinisation of the host cytoplasm. The excess negative charge in the host cytoplasm

would either be balanced by the protons produced during ammonia assimilation or transferred to carboxylic acids (Kirkby and Mengel 1967, Raven and Smith 1976). In this way the peribacteroid membrane ATPase may regulate both the uptake of carboxylic acids by the bacteroids and the export of ammonia to the nodule cytoplasm.

In order to test the hypothesis that nitrate affects nitrogenase activity by inhibiting carboxylic acid uptake across the peribacteroid membrane, it will be necessary, first of all, to isolate peribacteroid units (bacteroids enclosed by the peribacteroid membrane) and determine whether the peribacteroid membrane has a carboxylic acid transporter. Preliminary data suggests that malate, succinate and fumarate but not oxoglutarate, glutamate, pyruvate or arabinose are taken up across the peribacteroid membrane (Price, Day and Gresshoff, pers. comm.). Secondly, the effect of nitrate and/or products of its reduction on the activity of this transporter will need to be ascertained. Another approach may involve the characterisation of nodulins located in the peribacteroid membrane (Fortin et al. 1985, Katinkas and Verma 1985). One of these nodulins, nodulin-24 has already been partially characterised and is thought to be a transport protein (Blumwald et al. 1985, Fortin et al. 1985).

The roots of soybean plants dependent solely on N_2 as a nitrogen source take up an excess of cations over anions (Israel and Jackson 1982). The cations are exchanged for protons and as a result of this, excess hydroxyl ions are generated in the root cytoplasm. It is proposed that the negative charge associated with these hydroxyl ions is transferred to carboxylic acids, particularly malate (Israel and Jackson 1982). In other words, malate synthesis is promoted in the roots of N_2

dependent plants. In contrast, nitrate dependent soybean plants take up an excess of anions over cations (Israel and Jackson 1982).

On the basis of similar observations in other plant families, Ben Zioni et al. (1971) and Dijkshoorn (1971) proposed a model to explain how nitrate uptake by the roots may be regulated by nitrate reduction in the shoots. Nitrate was taken up by the roots and transported to the shoots in the xylem. In the shoots, nitrate reduction and the consequent generation of excess hydroxyl ions, was associated with malate synthesis. Some of this malate was transported, via the phloem, to the roots where it was decarboxylated to yield HCO_3^- which exchanged for nitrate in the growth medium. Briefly then, since nitrate reduction takes place predominantly in the shoots in soybean (see Pate 1983), N_2 dependent plants may tend to synthesize malate, whereas nitrate dependent plants may tend to degrade malate, in the root tissue. Therefore, if malate is the carbon source supplied by the host plant to the bacteroids, then nitrate-induced inhibition of nitrogenase activity may lead to a decline in the availability of respiratory substrates, due to the degradation of malate to provide a counter ion for nitrate uptake. Contrary to this hypothesis, however, there is no change in the level of malate in the nodules of soybean plants treated with nitrate for two days, despite a marked inhibition of nitrogenase activity (D.A. Day, pers. comm.). Furthermore, van Beuischem et al. (1985) have shown that recycling of malate from the shoot may not be required to provide counter-ions for nitrate uptake; at least in species which reduce nitrate predominantly in the roots and possibly also in species which reduce nitrate predominantly in the shoots.

Besides being dependent on their host plant for a carbon source, Kahn et al. (1985) have suggested that bacteroids may also require a nitrogen source, possibly in the form of glutamate. This hypothesis is based firstly on the observation that bacteroids express only low levels of GS, GOGAT and GDH activity and therefore probably do not assimilate significant quantities of the ammonia they produce (Brown and Dilworth 1975, Werner et al. 1980). Secondly, bacteroids export rather than import ammonia and they lack the ammonium uptake system of free-living Rhizobium bacteria (Bergersen and Turner 1967, O'Hara et al. 1985, Howitt et al. 1986). Lastly, Rhizobium mutants defective in glutamate catabolism form ineffective nodules (Kahn et al. 1985).

Based on these observations, Kahn et al. (1985) propose that the host plant regulates bacteroid proliferation and nitrogenase activity by controlling the supply of glutamate to the bacteroids. If this is so, then another way in which nitrate may inhibit nitrogenase activity may be by means of an inhibitory effect of products of nitrate reduction in the roots on GOGAT activity in the nodules. GOGAT purified from lupin nodules is inhibited by glutamate, oxaloacetate, aspartate and asparagine (Boland and Benny 1977). It should be noted, however, that the decline in nitrogenase activity of nodulated roots, in response to nitrate treatment, preceded the decline in GOGAT activity (this study) and therefore, decreased GOGAT activity was probably a result rather than the cause of the nitrate-induced decline in nitrogenase activity.

There is other evidence too which contradicts the Kahn et al. (1985) hypothesis. First and foremost, glutamate does not appear to be taken up across the peribacteroid membrane (Price, Day and Gresshoff, pers. comm.). Secondly, although Rhizobium mutants defective in

carboxylic acid uptake form ineffective nodules, the bacteroids within those nodules continue to divide and differentiate normally (see Ronson and Astwood 1985) suggesting that the low levels of ammonia assimilating enzymes in the bacteroids are sufficient to provide enough nitrogen for bacteroid growth.

Besides preceding the decline in extractable nodule GOGAT activity, the nitrate-induced decline in nitrogenase activity of nodulated roots also preceded any decline in (1) nitrogenase activity of isolated bacteroids, (2) extractable activity of some of the enzymes involved in ammonia assimilation and ureide biosynthesis in the nodules and (3) extractable activity of the enzymes involved in sucrose degradation and CO_2 fixation in soybean nodules (see the present study). These results indicate that any loss of the capacity of nodules to assimilate N_2 and to utilize photosynthate is not the primary cause of the nitrate induced decline in nitrogenase activity of nodulated roots.

They are also consistent with the previously reported biphasic nature of the inhibitory effect of nitrate on nitrogenase activity of clover (Carroll and Gresshoff 1983). During the first phase, nitrogenase activity was fully recoverable once nitrate was removed, whereas during the second phase activity was no longer recoverable. Similar results have been obtained with soybean (Gibson 1976, Noel et al. 1982, Streeter 1985 b), although these authors did not discuss the biphasic nature of the nitrate response. The results of the present study, along with the observation that nitrate inhibits ^{35}S incorporation into bacteroid and nodule proteins (Bisseling et al. 1978, Noel et al. 1982), indicate that this second phase may correspond to a decline in the net synthesis of bacteroid and nodule proteins.

In view of the apparently long turnover time of nodule proteins, it may be more profitable to examine the effect of nitrate treatment on nodule mRNA levels. This approach may reveal differential effects of nitrate treatment on nodule proteins sooner than an analysis of the levels of the various proteins present in the nodule at any given time after the commencement of nitrate treatment. Another approach would involve pulse labelling of nodule proteins with ^{35}S. This would reveal whether nitrate affected the synthesis of some nodule proteins more or less than others.

Besides addressing the nitrate-inhibition phenomenon, this study also revealed some interesting characteristics of the supernodulating, nitrate-tolerant soybean mutant nts382 which warrant further investigation. First of all, the low nitrogenase activity per g nodule fresh weight of nts382 was shown to be largely explicable by the low bacteroid protein content of the nodules of the mutant. However, the possibility of the presence of a barrier to O_2 diffusion in nts382 nodules cannot be discounted (see above).

Despite the fact that nitrogenase activity per plant of nts382 was not greater than that of Bragg, the level of ureides in the nodules, xylem sap and leaves of the mutant was several-fold higher. This was proposed to be attributable to increased ureide biosynthesis in the roots and/or the nodules of nts382.

Since ureides, in soybean nodules, are derived from purines (see Schubert and Boland 1984), the high level of these nitrogenous compounds in nts382 suggests that there may be an alteration in purine biosynthesis in the mutant. Cytokinins are also derived from purines (see Moore 1979). Therefore, cytokinin biosynthesis may be altered in

nts382 as well. Cytokinins have been implicated in nodule initiation (see Libbenga and Bogers 1974, see also Newcomb 1980) and therefore, an alteration in cytokinin biosynthesis may explain the supernodulation phenotype of nts382.

Silver-stained, two dimensional polyacrylamide gels, revealed a 24Kd protein which was more abundant in the cytoplasm of nts382 nodules than it was in Bragg nodules. Nitrate treatment had no effect on this protein and therefore it is probably not associated with the nitrate tolerance of nts382. It may, however, be associated with the supernodulation phenotype of nts382 and other alterations associated with this, such as elevated ureide biosynthetic activity. It will be interesting, therefore, to purify and characterise this protein. Antisera could be raised against it and used to localise it in the nodule. Nodulin antisera, available from other laboratories could be tested for cross-reactivity.

Although this study failed to identify the primary cause of the nitrate-induced decline in nitrogenase activity, it did show that the loss of the enzymic capacity of bacteroids to fix N_2 and of nodules to assimilate fixed nitrogen and metabolise carbon is not involved. Furthermore, it showed that the supernodulating, nitrate-tolerant soybean mutant nts382 may be a useful tool for examining the mechanism of the inhibitory effect of nitrate on nodulation and N_2 fixation.

Bibliography

Antoniw LD, JI Sprent 1978 Primary metabolites of Phaseolus vulgaris nodules. Phytochemistry 17:675-678.

Appleby CA 1984 Leghaemoglobin and Rhizobium respiration. Ann Rev Plant Physiol 35:443-478.

Appleby CA, FJ Bergersen 1980 Preparation and experimental use of leghaemoglobin. In FJ Bergersen, ed, Methods for Evaluating Biological Nitrogen Fixation. John Wiley and Sons Ltd, Chichester, pp 315-336.

Atkins CA, JS Pate, BJ Shelp 1984 a Effects of short-term N_2 deficiency on N metabolism in legume nodules. Plant Physiol 76:705-710.

Atkins CA, BJ Shelp, PJ Storer 1985 Purification and properties of inosine monophosphate oxidoreductase from nitrogen-fixing nodules of cowpea (Vigna unguiculata L. Walp). Arch Biochem Biophys 236:807-814.

Atkins CA, BJ Shelp, PJ Storer, JS Pate 1984 b Nitrogen nutrition and the development of biochemical functions associated with nitrogen fixation and ammonia assimilation of nodules on cowpea seedlings. Planta 162:327-333.

Auger S, D Baulcombe, DPS Verma 1979 Sequence complexities of the poly(A)-containing mRNA in uninfected soybean root and the nodule tissue developed due to the infection by Rhizobium. Biochem Biophys Acta 563:496-507.

Bach MK, WE Magee, RH Burris 1958 Translocation of photosynthetic products to soybean nodules and their role in nitrogen fixation. Plant Physiol 33:118-124.

Bauer WD 1981 Infection of legumes by rhizobia. Ann Rev Plant Physiol 32:407-449.

Becana M, PM Aparicio-Tejo, M Sanchez-Diaz 1984 Root nodule enzymes of ammonia assimilation from Medicago sativa as influenced by nitrate levels. J Plant Physiol 116:285-292.

Ben-Zioni A, Y Vaadia, SH Lips 1971 Nitrate uptake by roots as regulated by nitrate reduction products of the shoot. Plant Physiol 24:288-290.

Bergersen FJ 1980 Measurement of nitrogen fixation by direct means. In FJ Bergersen, ed, Methods for Evaluating Biological Nitrogen Fixation. John Wiley and Sons Ltd, Chichester, pp 65-110.

Bergersen FJ, GL Turner 1967 Nitrogen fixation by the bacteroid fraction of breis of soybean root nodules. Biochem Biophys Acta 141:507-515.

Betts JH 1986 Identification of soybean genotypes with increased nodulation and nitrogen fixation in high nitrate soil. Graduate Diploma of Science Thesis, Australian National University, Canberra, Australia

Bisseling T, RC van den Bos, A van Kammen 1978 The effect of ammonium nitrate on the synthesis of nitrogenase and the concentration of leghaemoglobin in pea root nodules induced by Rhizobium leguminosarum. Biochim Biophys Acta 539:1-11.

Blumwald E, MG Fortin, PA Rea, DPS Verma, RJ Poole 1985 Presence of a host-plasma membrane type H^+-ATPase in the membrane envelope enclosing the bacteroids in soybean root nodules. Plant Physiol 78:665-672.

Boland MJ, AG Benny 1977 Enzymes of nitrogen metabolism in legume nodules. Purification and properties of NADH dependent glutamate synthetase from lupin nodules. Eur J Biochem 79:355-362.

Boland MJ, DG Blevins, DD Randall 1983 Soybean nodule xanthine dehydrogenase: A kinetic study. Arch Biochem Biophys 222:435-441.

Boland MJ, KJF Farnden, JG Robertson 1980 Ammonia assimilation in nitrogen-fixing legume nodules. In WE Newton, WH Orme-Johnson, eds, Nitrogen Fixation Vol II: Symbiotic Associations and Cyanobacteria. University Park Press, Baltimore, pp 33-52.

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72:248-254.

Brown CM, MJ Dilworth 1975 Ammonia assimilation by Rhizobium cultures and bacteroids. J Gen Microbiol 86:39-48.

Carroll BJ 1980 Nitrate effect on nodulation and nitrogen fixation in white clover. Honours Thesis, Australian National University, Canberra, Australia.

Carroll BJ 1985 The plant contribution to the soybean-Rhizobium symbiosis. PhD thesis, Australian National University, Canberra, Australia.

Carroll BJ, PM Gresshoff 1983 Nitrate inhibition of nodulation and nitrogen fixation in white clover. Z Pflanzenphysiol 110:69-76.

Carroll BJ, PM Gresshoff 1986 Isolation and initial characterisation of constitutive nitrate reductase-deficient mutants NR328 and NR345 of soybean (Glycine max). Plant Physiol 81:572-576.

Carroll BJ, DL McNeil, PM Gresshoff 1985 a Isolation and properties of soybean (Glycine max (L.) Merr.) mutants that nodulate in the presence of high nitrate concentration. Proc Natl Acad Sci 82:4162-4166.

Carroll BJ, DL McNeil, PM Gresshoff 1985 b A supernodulation and nitrate-tolerant symbiotic (nts) soybean mutant. Plant Physiol 78:34-40.

Cataldo DA, M Haroon, LE Schrader, VL Youngs 1975 Rapid colourimetric determination of nitrate in plant tissue by nitration of salicyclic acid. Commun Soil Sci and Plant Anal 6:71-80.

Coker GT, KR Schubert 1981 Carbon dioxide fixation in soybean roots and nodules. I. Characterisation and comparison with N_2 fixation and composition of xylem exudate during early nodule development. Plant Physiol 67:691-696.

Copeland L, DD Harrison, JF Turner 1978 Fructokinase (fraction III) of pea seeds. Plant Physiol 62:291-294.

Copeland L, M Morell 1985 Hexose kinases from the plant cytosolic fraction of soybean nodules. Plant Physiol 79:114-117.

Coventry DR, Dilworth MJ 1976 Synthesis and turnover of leghaemoglobin in lupin root nodules. Biochem Biophys Acta 447:1-10.

Cullimore JV, PV Lea, BJ Miflin 1982 Multiple forms of glutamine synthetase in the plant fraction of Phaseolus root nodules. Israel J Bot 31:155-162.

Cullimore JV, AP Sims 1980 An association between photorespiration and protein catabolism: Studies with Chlamydomonas. Planta 150:392-396.

Dart PJ, FV Mercer 1965 The influence of ammonium nitrate on the fine structure of nodules of Medicago tribuloides. Desr. and Trifolium subterraneum L. Arch Mikrobiol 51:233-257.

Day DA, H Lambers, J Bateman, BJ Carroll, PM Gresshoff 1986 Growth comparisons of a supernodulating soybean (Glycine max L.) mutant and its wild type parent. Physiol Plant, in press.

Dazzo FB, AE Gardiol 1984 Host specificity in Rhizobium-legume interactions. In DPS Verma, TH Hohn, eds, Genes Involved in Microbe-Plant Interactions. Springer-Verlag, Wien, pp 3-31.

Delves AC, A Mathews, DA Day, AS Carter, BJ Carroll, PM Gresshoff 1986 Regulation of the soybean-Rhizobium nodule symbiosis by shoot and root factors. Plant Physiol, in press.

Deroche M, E Carrayal, E Jolivet 1983 Phosphoenolpyruvate carboxylase in legume nodules. Physiol Veg 21:1075-1081.

Dijkshoorn W 1971 Partition of ionic constituents between organs. In RM Samish, ed, Recent Advances in Plant Nutrition. Gordon and Breach Science Publishers, New York, pp 447-476.

Dilworth MJ 1966 Acetylene reduction by nitrogen fixing preparations from Clostridium pasteurianum. Biochim Biophys Acta 127:285-294.

Duke SH, GE Ham 1976 The effect of nitrogen addition on N_2 fixation and on glutamate dehydrogenase and glutamate synthase activities in nodules and roots of soybeans inoculated with various strains of R. japonicum. Plant Cell Physiol 17:1037-1044.

Eady RR, R Issack, K Kennedy, JR Postgate, HD Ratcliffe 1978 Nitrogenase synthesis in Klebsiella pneumoniae: Comparison of ammonium and O_2 regulation. J Gen Microbiol 104:277-285.

Fortin MG, M Zelechowska, DPS Verma 1985 Specific targeting of membrane nodulins to the bacteroid-enclosing compartment in soybean nodules. EMBO J 4:3041-3046.

Fuller F, PW Kunster, T Nguyen, DPS Verma 1983 Soybean nodulin genes: Analysis of cDNA clones reveals several major tissue-specific sequences in nitrogen fixing root nodules. Proc Natl Acad Sci 80:2594-2598.

Fuller F, DPS Verma 1984 Appearance and accumulation of nodulin mRNAs and their relationship to the effectiveness of root nodules. Plant Molecular Biology 3:21-28.

Gadal P 1983 Phosphoenolpyruvate carboxylase and nitrogen fixation. Physiol Veg 21:1069-1074.

Gibson AH 1976 Recovery and compensation by nodulated legumes to environmental stress. In PS Nutman, ed, Symbiotic Nitrogen Fixation in

Plants IBP Synthesis Vol 7, Cambridge University Press, London, England, pp 385-403.

Gibson AH, JE Harper 1985 Nitrate effect on nodulation of soybean by Bradyrhizobium japonicum. Crop Sci 25:497-501.

Gibson AH, JD Pagan 1977 Nitrate effects on the nodulation of legumes inoculated with nitrate reductase deficient mutants of Rhizobium. Planta 134:17-22.

Gordon AJ, GJA Ryle, DF Mitchell, CE Powell 1985 The flux of ^{14}C-labelled photosynthate through soyabean root nodules during N_2 fixation. J Exp Bot 36:756-769.

Gresshoff PM, AC Delves 1986 Plant genetic approaches to symbiotic nodulation and nitrogen fixation in legumes. In PJ King, TH Hohn, eds, Plant Gene Research, Vol 3, Springer Verlag, Wien, in press.

Groat RG, CP Vance 1981 Root nodule enzymes of ammonia assimilation in alfalfa (Medicago sativa L.). Plant Physiol 67:1198-1203.

Groat RG, CP Vance 1982 Root and nodule enzymes of ammonia-assimilation in two plant-conditioned symbiotically ineffective genotypes of alfalfa (Medicago sativa L.). Plant Physiol 69:614-618.

Harper JE, AH Gibson 1984 Differential nodulation tolerance to nitrate among legume species. Crop Sci 24:797-801.

Herridge DF 1977 Carbon and nitrogen nutrition of two annual legumes, PhD Thesis, University of Western Australia, Perth, Australia.

Herridge DF 1982 Relative abundance of ureides and nitrate in plant tissues of soybean as a quantitative assay of nitrogen fixation. Plant Physiol 70:1-6.

Herridge DF 1984 Effects of nitrate and plant development on the abundance of nitrogenous solutes in root bleeding and vacuum extracted exudates of soybean. Crop Sci 24:173-178.

Hirel, B, C Bauet, F Jacobs, D Layzell, B King, DPS Verma 1986 Regulation of glutamine synthetase genes in soybean root nodules. In DPS Verma, ed, The Proceedings of the Third International Symposium on the Molecular Genetics of Plant-Microbe Interactions. Martinus Nijhoff, Montreal, Canada, in press.

Houwaard F 1979 Effect of ammonium chloride and methionine sulfoximine on the acetylene reduction of detached root nodules of peas (Pisum sativum). Applied and Environmental Microbiology 37:73-79.

Houwaard F 1980 Influence of ammonium and nitrate nitrogen on nitrogenase activity of pea plants as affected by light intensity and sugar addition. Plant and Soil 54:271-282.

Howitt SM, MK Udvardi, DA Day, PM Gresshoff 1986 Ammonia transport in free-living and symbiotic Rhizobium sp ANU 289. J Gen Microbiol 132:257-261.

Hunter WJ 1983 Soybean root and nodule nitrate reductase. Physiol Plant 59:471-475.

Israel DW, WA Jackson 1982 Ion balance, uptake, and transport processes in N_2-fixing and nitrate and urea dependent soybean plants. Plant Physiol 69:171-178.

Kahn ML, J Kraus, JE Sommerville 1985 A model of nutrient exchange in the Rhizobium-legume symbiosis. In HJ Evans, PJ Bottomley, WE Newton, eds, Nitrogen Fixation Research Progress. Martinus Nijhoff, Dordrecht, pp 193-199.

Katinkas P, DPS Verma 1985 Nodulin-24 gene codes for a peptide of the peribacteroid membrane and was generated by tandem duplication of a sequence resembling an insertion element. Proc Natl Acad Sci 82:4152-4161.

Kennedy IR 1970 Kinetics of acetylene and CN^- reduction by the N_2 fixing system of Rhizobium lupini. Biochim Biophys Acta 222:135-144.

King BJ, DB Layzell, DT Canvin 1986 The role of dark carbon dioxide fixation in root nodules of soybean. Plant Physiol 81:200-205.

Kirkby EA, K Mengel 1967 Ionic balance in different tissues of the tomato plant in relation to nitrate, urea and ammonium nutrition. Plant Physiol 42:6-14.

Klucas RV, K Lee, L Saari, B Kipp-Erickson 1985 Factors affecting functional leghaemoglobin in legume nodules. In PW Ludden, JE Burris, eds, Nitrogen Fixation and CO_2 Metabolism. Elsevier Science Publishing Co., pp 13-20.

Kouchi H, T Yoneyama 1984 a Dynamics of carbon photosynthetically assimilated in nodulated soya bean plants under steady-state conditions. 1. Development and application of $^{13}CO_2$ assimilation system at a constant ^{13}C abundance. Ann Bot 53:875-882.

Kouchi H, T Yoneyama 1984 b Dynamics of carbon photosynthetically assimilated in nodulated soya bean plants under steady-state conditions. 2. The incorporation of ^{13}C into carbohydrates, organic acids, amino acids and some storage compounds. Ann Bot 53:883-896.

Kouchi H, K Nakaji, T Yoneyama, J Ishizuka 1985 Dynamics of carbon photosynthetically assimilated in nodulated soya bean plants under steady-state conditions. 3. Time course study on ^{13}C incorporation into soluble metabolites and respiratory evolution of $^{13}CO_2$ from roots and nodules. Ann Bot 56:333-346.

Laane C, W Krone, W Konings, H Haaker, C Veeger 1980 Short-term effect of ammonium chloride on nitrogen fixation by Azotobacter

vinelandii and by bacteroids of Rhizobium leguminosarum. Eur J Biochem 103:39-46.

Laemmli UK, M Favre 1973 Maturation of the head of bacteriophage T4. I. DNA packaging events. J Mol Biol 80:575-599.

Laing WA, JT Christeller, WD Sutton 1979 Carbon dioxide fixation by lupin root nodules. II. Studies with ^{14}C-labelled glucose, the pathway of glucose catabolism, and the effects of some treatments that inhibit nitrogen fixation. Plant Physiol 63:450-454.

Larsen K, BU Jochimsen 1986 Expression of nodule specific uricase in soybean callus tissue is regulated by O_2. EMBO J 5:15-19.

Latimore M, J Giddens, DA Ashley 1977 Effect of ammonium and nitrate nitrogen upon photosynthate supply and nitrogen fixation by soybeans. Crop Sci 17:399-404.

Lawrie A, CT Wheeler 1973 The supply of photosynthetic assimilates to nodules of Pisum sativum L. in relation to the fixation of nitrogen. New Phytologist 72:1341-1348.

Legocki RP, DPS Verma 1979 A nodule specific plant protein (Nodulin-35) from soybean. Science 205:190-193.

Legocki RP, DPS Verma 1980 Identification of "nodule-specific" host proteins (nodulins) involved in the development of Rhizobium-legume symbiosis. Cell 20:153-163.

Lew RR and RM Spanswick 1985 Characterisation of anion effects on the nitrate-sensitive ATP-dependent proton pumping activity of soybean (Glycine max L.) seedling root microsomes. Plant Physiol 77:352-357.

Libbenga KR, RJ Bogers 1974 Root-nodule morphogenesis. In A Quispel, ed, The Biology of Nitrogen Fixation. North-Holland Publishing Co, Amsterdam, Holland, pp 430-472.

Lowry OH, NJ Rosebrough, AL Farr, RJ Randall 1951 Protein measurement with the folin reagent. J Biol Chem 193:265-275.

Lucas K, MJ Boland, KR Schubert 1983 Uricase from soybean root nodules: Purification, properties and comparison with the enzyme from cowpea. Arch Biochem Biophys 226:190-197.

McClure PR, DW Israel 1979 Transport of nitrogen in the xylem of soybean plants. Plant Physiol 64:411-416.

McClure PR, DW Israel, RJ Volk 1980 Evaluation of the relative ureide content of xylem sap as an indicator of N_2 fixation in soybeans. Plant Physiol 66:720-725.

McKay IA, AR Glenn, MJ Dilworth 1985 Gluconeogenesis in Rhizobium leguminosarum MNF3841. J Gen Microbiol 131:2067-2073.

McNeil DL, BJ Carroll, PM Gresshoff 1984 The nitrogen fixation capacity of bacteroids extracted from soybean nodules inhibited by nitrate, ammonia or dark treatments. In BS Ghai, ed, Symbiotic Nitrogen Fixation. USG Publishers and Distributors, Ludhiana, India, pp 79-88.

Mellor RB, TMIE Christensen, D Werner 1986. Choline kinase II is present only in nodules that synthesize stable peribacteroid membranes. Proc Natl Acad Sci 83:659-663.

Minchin FR, JF Witty, JE Sheehy, M Muller 1983 A major error in the acetylene reduction assay: Decreases in nodular nitrogenase activity under assay conditions. J Exp Bot 34:641-649.

Moore TC 1979 Biochemistry and Physiology of Plant Hormones. Springer-Verlag, New York.

Morrell M, L Copeland 1984 Enzymes of sucrose breakdown in soybean nodules: Alkaline invertase. Plant Physiol 74:1030-1034.

Morrell M, L Copeland 1985 Sucrose synthase of soybean nodules. Plant Physiol 78:149-154.

Munns DN 1968 Nodulation of Medicago sativa in solution culture. IV. Effects of indole-3-acetate in relation to acidity and nitrate. Plant Soil 29:257-261.

Nelson RS, SA Ryan, JE Harper 1983 Soybean mutants lacking constitutive nitrate reductase activity. I. Selection and initial plant characterisation. Plant Physiol 72:503-509.

Newcomb W 1980 Control of morphogenesis and differentiation of pea root nodules. In WE Newton, WH Orme-Johnson, eds, Nitrogen Fixation Vol II, University Park Press, Baltimore, pp 87-102.

Nguyen J, J Vidal, C Perrot-Rechenmann, P Gadal 1986 Immunochemical studies on xanthine dehydrogenase of soybean root nodules. Planta 167:190-195.

Noel KD, M Carneol, WJ Brill 1982 Nodule protein synthesis and nitrogenase activity of soybeans exposed to fixed nitrogen. Plant Physiol 70:1236-1241.

O'Farrell PH 1975 High resolution two dimensional electrophoresis of proteins. J Biol Chem 250:4007-4021.

Oghoghorie CGO, Pate JS 1971 The nitrate stress syndrome of the nodulated field pea (Pisum arvense L.). In TA Lie, EG Muller, eds, Biological Nitrogen Fixation in Natural and Agricultural Habitats. Plant and Soil, special volume, pp 185-202.

O'Hara GW, IT Riley, AR Glenn, MJ Dilworth 1985 The ammonium permease of Rhizobium leguminosarum MNF3841. J Gen Microbiol 131:757-764.

Ohyama T, K Kumazawa 1979 Incorporation of ^{15}N into various nitrogenous compounds in intact soybean nodules after exposure to $^{15}N_2$ gas. Soil Sci Plant Nutr 24:525-533.

Pate JS 1983 Patterns of nitrogen metabolism in higher plants and their ecological significance. In JA Lee, S McNeill, IH Rorison, eds, Nitrogen as an Ecological Factor. Blackwell, Oxford, pp 225-255.

Pate JS, CA Atkins 1983 Nitrogen uptake, transport and utilization. In WJ Broughton, ed, Nitrogen Fixation Volume 3: Legumes. Oxford University Press, New York, pp 245-298.

Pate JS, BES Gunning, LS Briarty 1969 Ultrastructure and functioning of the transport system of the leguminous root nodule. Planta 85:11-34.

Pfeiffer NE, NSA Malik, FW Wagner 1983 Reversible dark-induced senescence in soybean root nodules. Plant Physiol 71:393-399.

Polayes, DA, KR Schubert 1984 Purine synthesis and catabolism in soybean seedlings. Plant Physiol 75:1104-1110.

Rainbird RM, CA Atkins 1981 Purification and properties of urate oxidase from nitrogen-fixing nodules of cowpea. Biochem Biophys Acta 659:132-140.

Raven JA, FA Smith 1976 Nitrogen assimilation and transport in vascular land plants in relation to intracellular pH regulation. New Phytol 76:415-423.

Reibach PH, JG Streeter 1983 Metabolism of ^{14}C-labelled photosynthate and distribution of enzymes of glucose metabolism in soybean nodules. Plant Physiol 72:634-640.

Reynolds PHS, DG Blevins, DD Randall 1984 5-Phosphoribosylpyrophosphate amidotransferase from soybean root nodules: Kinetic and regulatory properties. Arch Biochem Biophys 229:623-631.

Rigaud J, A Puppo 1977 Effect of nitrite upon leghaemoglobin and interaction with nitrogen fixation. Biochem Biophys Acta 497:702-706.

Ronson CW, PA Astwood 1985 Genes involved in the carbon metabolism of bacteroids. In HJ Evans, PJ Bottomley, WE Newton, eds, Nitrogen Fixation Research Progress. Martinus Nijhoff, Dordrecht, pp 201-207.

Ryan SA, RS Nelson, JE Harper 1983 Soybean mutants lacking constitutive nitrate reductase activity. II. Nitrogen assimilation, chlorate resistance, and inheritance. Plant Physiol 72:510-514.

Sammons DW, LD Adams, EE Nishizawa 1981 Ultrasensitive silver-based colour staining of polypeptides in polyacrylamide gels. Electrophoresis 2:135-141.

Sandeman RA, PM Gresshoff 1985 Nitrogenase activity and inactivation in isolated bacteroids from the legume siratro and the non-legume Parasponia rigida. Plant Sci Lett 37:199-204.

Scholl RL, JE Harper, RH Hageman 1974 Improvements of the nitrite colour development in assays of nitrate reductase by phenazine methosulfate and zinc acetate. Plant Physiol 53:825-828.

Schollhorn R, RH Burris 1966 Study of intermediates in nitrogen fixation. Federation Proc (abstr) 25:710.

Schubert KR 1981 Enzymes of purine biosynthesis and catabolism in Glycine max. Plant Physiol 68:1115-1122.

Schubert KR 1986 Products of biological nitrogen fixation in higher plants: Synthesis, transport, and metabolism. Ann Rev Plant Physiol 37:539-574.

Schubert KR, MJ Boland 1984 The cellular and intracellular organization of the reactions of ureide biogenesis in nodules of tropical legumes. In C Veeger, WE Newton, eds, Advances in Nitrogen Fixation Research. Nijhoff/Junk, Pudoc, pp 445-451.

Sen D, HM Schulman 1980 Enzymes of ammonia assimilation in the cytosol of developing soybean root nodules. New Phytol 85:243-250.

Sheehy JE, FR Minchin, JF Witty 1983 Biological control of the resistance to O_2 flux in nodules. Annals of Botany 52:565-571.

Shelp BJ, RJ Ireland 1985 Ureide metabolism in leaves of nitrogen-fixing soybean plants. Plant Physiol 77:779-783.

Small JGC, OA Leonard 1969 Translocation of C^{14}-labelled photosynthate in nodulated legumes as influenced by nitrate nitrogen. Amer J Bot 56:187-194.

Stephens BD, CA Neyra 1983 Nitrate and nitrite reduction in relation to nitrogenase activity in soybean nodules and Rhizobium japonicum bacteroids. Plant Physiol 71:731-735.

Streeter JG 1979 Allantoin and allantoic acid in tissues and stem exudate from field grown soybean plants. Plant Physiol 63:478-480.

Streeter JG 1981 Effect of nitrate in the rooting medium on carbohydrate composition of soybean nodules. Plant Physiol 68:840-844.

Streeter JG 1985 a Nitrate inhibition of legume nodule growth and activity. I. Long term studies with a continuous supply of nitrate. Plant Physiol 77:321-324.

Streeter JG 1985 b Nitrate inhibition of legume nodule growth and activity. II. Short term studies with high nitrate supply. Plant Physiol 77:325-328.

Streit L, Nelson RS, Harper JE 1985 Nitrate reductases from wild-type and nr_1-mutant soybean (Glycine max [L.] Merr.) leaves. I. Purification, kinetics and physical properties. Plant Physiol 78:80-84.

Thomas RJ, LE Schrader 1981 a The assimilation of ureides in shoot tissues of soybeans. Plant Physiol 67:973-976.

Thomas RJ, LE Schrader 1981 b Ureide metabolism in higher plants. Phytochemistry 20:361-371.

Thummler F, DPS Verma 1986 Nodulin-100 gene of soybean codes for nodule-specific, sucrose synthetase. In DPS Verma, ed, The Proceedings of the Third International Symposium on the Molecular Genetics of Plant-Microbe Interactions. Montreal, Canada, Martinus Nijhoff, in press.

Trinchant JC, AM Birot, J Rigaud 1981 O_2 supply and energy-yielding substrates for nitrogen fixation (acetylene reduction) by bacteroid preparations. J Gen Microbiol 125:159-165.

Trinchant JC, J Rigaud 1980 Nitrite inhibition of nitrogenase from soybean bacteroids. Arch Microbiol 124:49-54.

Trinchant JC, J Rigaud 1984 Nitrogen fixation in French-beans in the presence of nitrate: Effect on bacteroid respiration and comparison with nitrite. J Plant Physiol 116:209-217.

Triplett EW 1985 Intercellular nodule localisation and nodule specificity of xanthine dehydrogenase in soybean. Plant Physiol 77:1004-1009.

Triplett EW, DG Blevins, DD Randall 1982 Purification and properties of soybean nodule xanthine dehydrogenase. Archiv Biochem Biophys 219:39-46.

Truchet GL, FB Dazzo 1982 Morphogenesis of root nodules incited by Rhizobium meliloti in the presence of combined nitrogen. Planta 154:352-360.

Valera CL, M Alexander 1965 Reversal of nitrate inhibition of nodulation by indole-3-acetic acid. Nature 206:326-331.

van Beuischem ML, R Baas, EA Kirkby, JA Nelemans 1985 Intracellular pH regulation during NO_3^- assimilation in shoots and roots of Ricinus communis. Plant Physiol 78:768-773.

Vance CP, KLM Boylan, CA Maxwell, GH Heichel, LL Hardman 1985 Transport and partitioning of CO_2 fixed by root nodules of ureide and amide producing legumes. Plant Physiol 78:774-778.

Verma DPS, H Bergmann, F Fuller, E Preddie 1983 The role of plant genes in soybean-Rhizobium interactions. In A Puhler, ed, Molecular Genetics of the Bacteria-Plant Interaction. U Springer-Verlag, Berlin, Heidelberg, pp 156-163.

Vogels GD, C van der Drift 1970 Differential analyses of glyoxylate derivatives. Analytical Biochemistry 33:143-157.

Wasfi M, J Prioul 1986 A comparison of inhibition of French-beans and soybean nitrogen fixation by nitrate, 1% O_2 or direct assimilate deprivation. Physiol Plant 66:481-490.

Werner D, E Morschel, R Striplf, B Winchenbach 1980 Development of nodules of Glycine max infected with an ineffective strain of Rhizobium japonicum. Planta 147:320-329.

Whitmore-Smith D 1985 Nitrate metabolism in nitrate reductase deficient soybean mutants. Honours thesis, Australian National University, Canberra, Australia.

Winkler RG, JC Polacco, DG Blevins, DD Randall 1985 Enzymic degradation of allantoate in developing soybeans. Plant Physiol 79:787-793.

Witty JF, FR Minchin, JE Sheehy, M Ines Minguez 1984 Acetylene-induced changes in the O_2 diffusion resistance and nitrogenase activity of legume root nodules. Ann Bot 53:13-20.

Wong PP, HJ Evans 1971 Poly-β-hydroxybutyrate utilization by soybean (Glycine max Merr.) nodules and assessment of its role in maintenance of nitrogenase activity. Plant Physiol 47:750-755.

Yemm EW, EC Cocking 1955 The determination of amino acids with ninhydrin. Analyst 80:209-213.

Zengbe M, P Tillard, J Cleyet-Marel, L Salsac 1984 Influence de la souche de Rhizobium et de l'azote mineral sur la synthese des ureides chez le Soja. Physiol Veg 22:285-294.